

## I. Kinase/ATPase Activity Assays

### A. Luminescent ATP/ADP Detection Assays

#### ADP-Glo™ Kinase Assay Family

The ADP-Glo™ Kinase Assay (Cat.# V9101) is a luminescent kinase assay that measures ADP formed from a kinase reaction; ADP is converted into ATP, which is converted into light by Ultra-Glo™ Luciferase. The luminescent signal positively correlates with kinase activity. The assay is well suited for measuring the effects chemical compounds have on the activity of a broad range of purified kinases, making it ideal for both primary screening as well as kinase selectivity profiling. The ADP-Glo™ Kinase Assay can be used to monitor the activity of virtually any ADP-generating enzyme (e.g., kinase or ATPase) using up to 1mM ATP. The ADP-Glo™ Max Assay (Cat.# V7001) can be used when higher concentrations (up to 5mM) are required.

The assay is performed in two steps; first, after the kinase reaction, an equal volume of ADP-Glo™ Reagent is added to terminate the kinase reaction and deplete the remaining ATP. In the second step, the Kinase Detection Reagent is added, which simultaneously converts ADP to ATP and allows the newly synthesized ATP to be measured using a coupled luciferase/luciferin reaction (Figure 7.1).

The ADP-Glo™ Kinase Assay has a high dynamic range and produces a strong signal at low ATP to ADP conversion, making it well suited for screening low activity kinases such as growth factor receptor tyrosine kinases. The assay produces minimal false hits and Z' values of greater than 0.7.

The assay can be performed over a wide range of ATP concentrations (low micromolar to millimolar). This allows detection of small concentrations of ADP in the presence of large amounts of ATP (Figure 7.2), producing very high signal-to-background (SB) ratios (Figure 7.2). The robustness of the ADP-Glo™ Kinase Assay and suitability for high-throughput applications is evidenced by high Z'-factor values reported in previous studies (Tai *et al.* 2011). The ADP-Glo™ Kinase Assay is as sensitive as radioactivity-based methods and more sensitive than fluorescence-based technologies (Tai *et al.* 2011; Zegzouti *et al.* 2009; Vidugiriene *et al.* 2009). In order to lower the background and further improve the sensitivity of the assay, we increased the purity of our ATP to have less ADP contamination. To assess the importance of ATP purity on ADP-Glo™ assay sensitivity, we compared the signal-to-background ratios generated in an ADP-Glo™ assay using Promega Ultra Pure ATP and ATP from other suppliers. The Promega ATP outperforms ATP from other sources by greatly improving ADP-Glo™ assay sensitivity with SB ratios that are 2–3 times higher than those produced using other commercial preparations (Zegzouti *et al.* 2011).

#### Before You Begin

##### Materials Required:

- ADP-Glo™ Assay (Cat.# V9101, V9102, V9103) or ADP-Glo™ Max Assay (Cat.# V7001, V7002) and appropriate Protocol (Technical Manual #TM313 or TM343).
- solid white multiwell plates (do not use black plates)
- multichannel pipet or automated pipetting station
- plate shaker
- luminometer capable of reading multiwell plates
- appropriate substrate
- ADP-producing enzyme (e.g., ATPase or kinase)

##### General Instructions for Preparing Detection Buffer

1. Thaw the Detection Buffer at room temperature, and look for any precipitate.
2. If a precipitate is present, incubate the Detection Buffer at 37°C with constant swirling for 15 minutes.

##### General Instructions for Detection Reagent Preparation

1. Equilibrate the Detection Buffer and the Detection Substrate to room temperature before use.
2. Transfer the entire volume of Detection Buffer into the amber bottle containing the Detection Substrate to reconstitute the lyophilized substrate. This forms the Detection Reagent.
3. Mix by gently vortexing, swirling or inverting the contents to obtain an homogeneous solution. The Detection Substrate should go into solution in less than one minute.
4. The Detection Reagent should be used immediately or dispensed into aliquots and stored at –20°C.

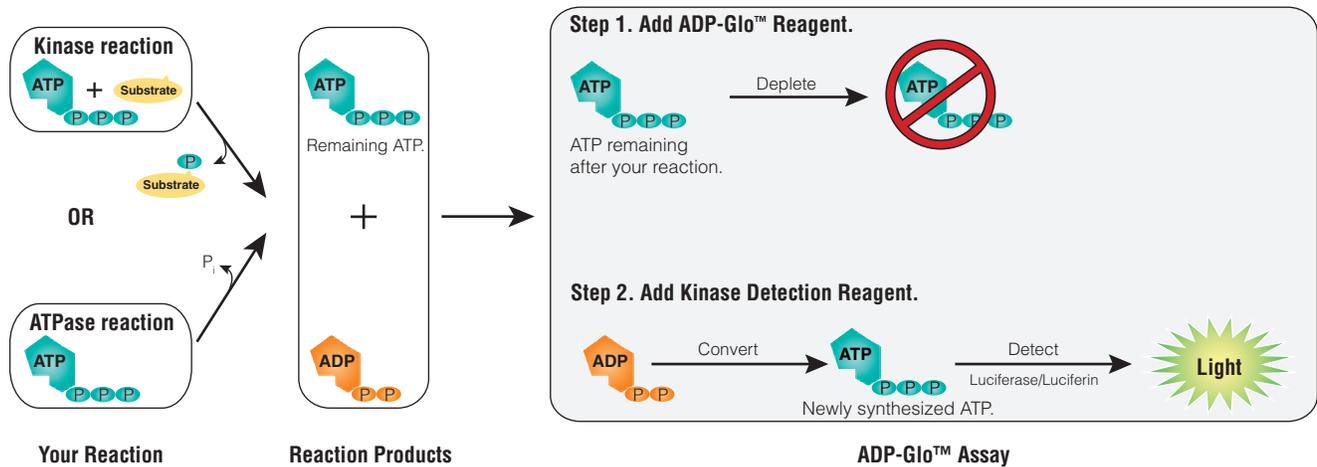
##### Generating a Standard Curve for the Conversion of ATP to ADP

1. To estimate the amount of ADP produced in the reaction, we recommend creating a standard curve that represents the luminescence corresponding to the conversion of ATP to ADP (the "ATP-to-ADP" conversion curve") based on the ATP concentration used in the kinase or ATPase reaction. These standard curves represent the amounts of ATP and ADP available in a reaction at the specified conversion percentage (Table 7.1). The standard samples used to generate an ATP-to-ADP standard are created by combining the appropriate volumes of ATP and ADP stock solutions. For more information on generating standard curves see Technical Manual TM313 for the ADP-Glo™ Kinase Assay or Technical Manual TM343 for the ADP-Glo™ Max Assay and the Kinase Titration and Determination of SB10 (Part A) below.

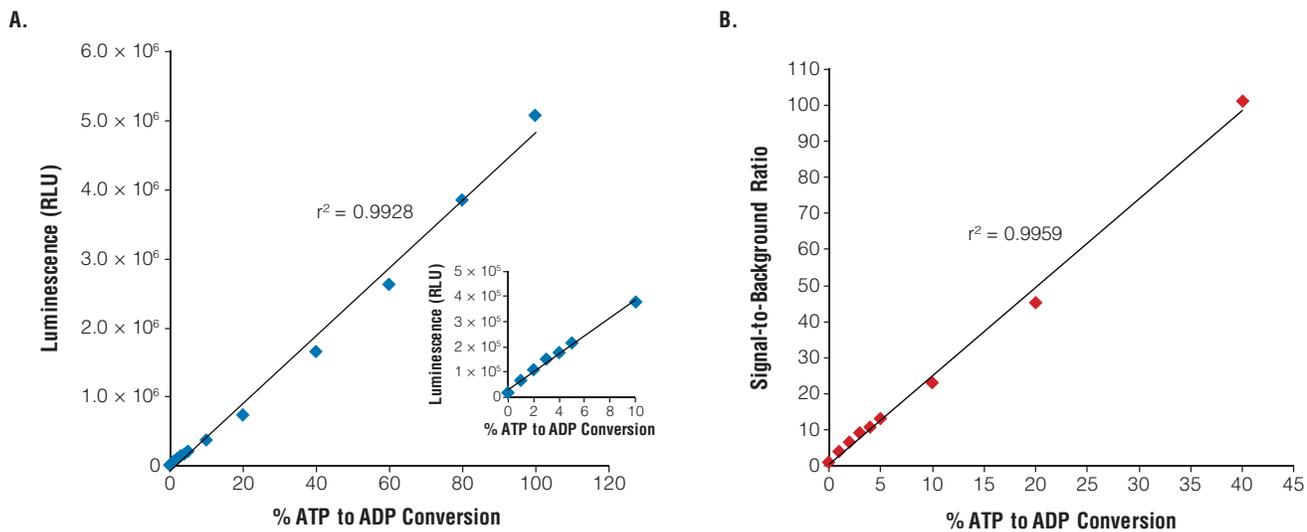
##### General Overview of ADP-Glo™ Kinase Assay Protocol

1. Perform a kinase reaction using 1X kinase buffer. (See appropriate Technical Manual for details.)

# Kinase Enzyme System Protocol



**Figure 7.1. The ADP-Glo™ Assay Principle.** The assay is composed of two steps. After the kinase or ATPase reaction, the first step is performed by addition of the ADP-Glo™ Reagent that terminates the kinase reaction and depletes any remaining ATP (40-minute incubation time). Addition of a second reagent converts ADP to ATP and generates light from the newly synthesized ATP using a luciferase/luciferin reaction (incubation is 30–60 minutes depending on the ATP concentration used in the kinase reaction). The light generated is proportional to ADP present and, consequently, kinase or ATPase activity. The assay is performed at room temperature and is compatible with automation.



**Figure 7.2. Linearity and sensitivity of the ADP-Glo™ Kinase Assay.** The 1mM ATP-to-ADP percent conversion curve (standard curve) was prepared in 1X reaction buffer A (40mM Tris [pH 7.5], 20mM MgCl<sub>2</sub>, and 0.1mg/ml BSA) without kinase present as described in Technical Manual #TM313. The standards were created by combining the appropriate volumes of ATP and ADP 1mM stock solutions. Five microliters of each ATP + ADP standard was transferred to a white, opaque 384-well plate. The ADP-Glo™ Kinase Assay was performed by adding 5µl of ADP-Glo™ Reagent and 10µl of Kinase Detection Reagent at room temperature to each well. ADP-Glo™ assay reagents were dispensed in 384-well plates using Multidrop® Combi nL liquid dispenser (Thermo Fisher Scientific). Luminescence values represent the mean of four replicates (RLU = relative light units). **Panel A.** Linearity of the assay up to 1mM ADP. **Panel B.** Sensitivity of the assay is shown as signal-to-background ratios (SB) over a wide range of % ATP-to-ADP conversion.

**Table 7.1. Percent Conversion of ATP to ADP Represented by the Standard Curve**

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12
%ADP	100	80	60	40	20	10	5	4	3	2	1	0
%ATP	0	20	40	0	80	90	95	96	97	98	99	100

2. Add ADP-Glo™ Reagent to stop the kinase reaction and deplete the unconsumed ATP, leaving only ADP and a very low background of ATP.
3. Incubate at room temperature for 40 minutes.
4. Add Detection Reagent to convert ADP to ATP and introduce luciferase and luciferin to detect ATP.
5. Incubate at room temperature for 30–60 minutes.

6. Measure the luminescence with a plate-reading luminometer or charge-coupled device (CCD) camera.

This is a general protocol. Please see the appropriate Technical Manuals for specific details and notes. To screen for inhibitors or to determine IC<sub>50</sub> values of kinase inhibitors using the ADP-Glo™ Kinase Assay Systems, see Technical Manual [TM313](#) for the ADP-Glo™ Kinase Assay or Technical Manual [TM343](#) for the ADP-Glo™ Max Assay.

### Sample Protocol for a Kinase Inhibitor (Staurosporine) Dose-Response Curve Using the ADP-Glo™ Assay

A kinase titration will be performed in order to determine the optimal amount of enzyme to use in subsequent inhibitor dose-response curve determination. To estimate the amount of ADP produced in a kinase reaction, create an ADP standard curve, named "ATP-to-ADP Conversion Curve". This curve represents the luminescence (RLU) corresponding to each % conversion of ATP-to-ADP based on the ATP concentration used in the kinase reaction. The standard samples used to generate an ATP-to-ADP conversion curve are created by combining the appropriate volumes of ATP and ADP stock solutions. **Kinase Titrations and ATP-to-ADP conversion curves** for similar ATP concentrations will be performed in one plate.

The percent ADP produced by each amount of enzyme is calculated using the reference RLUs from the conversion curves. By titrating the kinase, we will determine *SB10 value*, which corresponds to the amount of the kinase needed to generate a percent conversion reflecting the initial rate of the reaction. Usually we choose 5–10% conversion, as the signal-to-background ratio generated is higher than tenfold.

Using the SB10 amount of the kinase, we will perform a kinase inhibitor (staurosporine) dose response curve to calculate the IC<sub>50</sub> and to check for any ATPase contaminating activity that will not be inhibited.

### Reaction Buffers Needed Using 5X Reaction Buffer A:

5X Reaction Buffer A: 200mM Tris [pH 7.5], 100mM MgCl<sub>2</sub> and 0.5mg/ml BSA

4X Kinase Buffer: 4X Reaction Buffer A + 200μM DTT + (4X of any cofactors, e.g. MnCl<sub>2</sub>)

4X Kinase Buffer D made accordingly by adding 4% DMSO

1X Kinase Buffer made by diluting the 4X Kinase Buffer

1X Kinase Buffer D made by diluting 4X Kinase Buffer D

1X Kinase Buffer (5% DMSO) made by diluting the 4X Kinase Buffer and adding 5% DMSO

**Note:** All volumes described here are for duplicate samples. If you need to perform more than two replicates per sample, recalculate the volumes accordingly.

All steps are performed at room temperature (22–25°C).

### Part A: Kinase Titration and Determination of SB10 Generation of ATP-to-ADP Conversion Curves

1. In a 96-well plate, generate the ATP-ADP series needed by diluting in 1X Kinase Buffer D the samples from a 10X concentrated ATP + ADP ranges as described below.
2. **Preparing 10X Conversion Curve Standards:** Prepare 10X ADP/ATP stock plates in water as described in the tables below to make 100μl stock solutions of ATP/ADP standards (Table 7.2). You will need 1ml of your 10X ATP and 500μl of the 10X ADP **Note:** If you are working with only one ATP concentration, make only the corresponding 10X stocks.
3. **ATP Stock Solution Preparation (starting with a 1mM solution)**

Final conc. desired	Prepare this 10X stock	ATP (μl)	Water (μl)
1μM	10μM	10	990
5μM	50μM	50	950
10μM	100μM	100	900

### ADP Stock Solution Preparation (starting with a 1mM solution)

Final conc. desired	Prepare this 10X stock	ADP (μl)	Water (μl)
1μM	10μM	5	495
5μM	50μM	25	475
10μM	100μM	50	450

### ATP Stock Solution Preparation (starting with a 10mM solution)

Final conc. desired	Prepare this 10X stock	ATP (μl)	Water (μl)
100μM	1.0mM	100	900
250μM	2.5mM	250	750
500μM	5mM	500	500

### ADP Stock Solution Preparation (starting with a 10mM solution)

Final conc. desired	Prepare this 10X stock	ADP (μl)	Water (μl)
100μM	1.0mM	50	450
250μM	2.5mM	125	375
500μM	5mM	250	250

4. After you have prepared your ATP and ADP stock solutions, create a conversion curve 10X by transferring the amounts of each solution as described in Table 7.2.
5. **Important Note:** Use the remaining 100% ATP from your conversion curve plate to run the kinase reaction in order to have similar background levels.
6. Preparing a 1X ADP/ATP working dilution plate in 1X kinase reaction buffer: Mix 105μl of 4X Kinase Buffer D with 273μl of water. Transfer 27μl/well to a 96-well

**Table 7.2. 10X Conversion Curve Preparative Plate**

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10	Well 11	Well 12
% Conversion	100	80	60	40	20	10	5	4	3	2	1	0
ADP (μl)	100	80	60	40	20	10	5	4	3	2	1	0
ATP (μl)	0	20	40	0	80	90	95	96	97	98	99	100

plate, then transfer 3μl of the 10X ATP/ADP curve to each respective well in the dilution plate. This will give a final volume of 30μl, sufficient for 4 replicates.

### Preparation of Kinase Titration Components:

1. Prepare the kinase titrations at the same ATP concentrations as ATP-to-ADP conversion curves.
2. **Substrate Mix Preparation:** For each kinase, prepare 200μl of 2.5X ATP/Substrate Mix in a 1.5ml tube. Use the 10μM examples below for a guideline. **Note:** Use ATP from the same 10X ATP that you used for the conversion curve.

### Substrate Mix Preparation (10μM example)

Component	Amount
4X Kinase Buffer D	50μl
100μM ATP (10X)	50μl
Substrate (1mg/ml)	100μl

### Substrate Mix Preparation: If the substrate is MBP, Casein or Histone H1:

Component	Amount
4X Kinase Buffer D	50μl
100μM ATP (10X)	50μl
Water	50μl
Substrate (1mg/ml)	50μl

3. Transfer 14μl of 2.5X ATP/Substrate Mix to odd numbered wells (1,3,5...23) of a 384-well plate in **Row X**. This is your *ATP/Substrate preparative row*.
4. **Enzyme Dilution Preparation:** Add 10μl of 1X Kinase Buffer D to odd numbered wells, starting with well 3 (3, 5, 7...23) of the 384-well plate in **Row Y**. Do not add buffer to well 1. This is your *Kinase Dilution preparative row*.
5. Prepare 20μl kinase solution as described in the table below (3μl/reaction/well). This will give 200ng kinase/3μl starting concentration.

### Kinase Solution Preparation

Component	Volume
Water	1.67μl
4X Kinase Buffer D	5μl
Kinase (100ng/μl)	13.33μl

6. Add 20μl of Kinase Solution to well 1 of the *Kinase Dilution preparative row Y*. From there, prepare a 1:1 serial dilution of the kinase as shown in Figure 7.3. Mix well after each dilution by pipetting before transferring 10μl

to the next well. Do not continue the serial dilution after well 21. **Note:** Do not create bubbles while preparing the dilution series.

### 7. Kinase Reaction and Conversion Curve Experiment:

Transfer 5μl of the diluted ATP-ADP series in replicates from your *1X ADP/ATP working dilution plate* into the wells of your 384-well assay plate that are designated for the conversion curve.

8. Transfer 3μl of kinase samples in duplicates from the wells of the *kinase titration preparative, Row Y* to the wells of the assay plate designated for the kinase reactions.

9. Transfer 2μl of the corresponding 2.5X ATP/Substrate Mix from the wells of the *ATP/Substrate preparative Row X* to the same assay rows where the kinase dilutions are.

10. Spin the plate. Mix with a plate shaker for 2 minutes. Incubate the reaction at room temperature for 60 minutes or the desired time.

11. **ADP detection with ADP-Glo™ Kinase Assay:** After the kinase reaction incubation is complete, add 5μl of ADP-Glo™ Reagent to all wells in your assay plate. Mix for 2 minutes and incubate at room temperature for 40 minutes.

12. Add 10μl of kinase detection reagent to all wells in your assay plate. Mix for 2 minutes and then incubate at room temperature for 30–60 minutes.

13. Measure the luminescence (integration time, 0.5 second).

14. Calculate the *SB10 value* (ng or nM). *SB10* is the amount needed to generate a 5–10% ATP to ADP conversion (usually this kinase amount generates a signal-to-background ratio of greater than tenfold).

### Part B: Staurosporine Inhibitor Dose Response Curve

1. **Preparation of inhibitor titration components:** Add 50μl of 1X Kinase Buffer (with 5% DMSO) to wells A2–B12 of a 96-well plate. These are your *inhibitor titration preparative rows*. **Note:** Do not add buffer to well A1.

2. Prepare 100μl of 50μM staurosporine solution (will be 5% DMSO) as described in the table below (final 1μl/reaction/well). This will give 10μM staurosporine (1% DMSO) starting concentration in the assay.

Well #	Kinase	Starting Volume of Each Well	Volume to Transfer
1	200ng	20µl	10µl
3	100ng	10µl	10µl
5	50ng	10µl	10µl
7	25ng	10µl	10µl
9	12.5ng	10µl	10µl
11	6.25ng	10µl	10µl
13	3.12ng	10µl	10µl
15	1.56ng	10µl	10µl
17	0.78ng	10µl	10µl
19	0.39ng	10µl	10µl
21	0.1953ng	10µl	0µl
23	0	10µl	No transfer, buffer only

Figure 7.3. Performing serial 1:1 dilutions of kinase.

### Staurosporine Solution Preparation

Component	Volume
Water	70µl
4X Kinase Buffer	25µl
Staurosporine in DMSO (1mM)	5µl

3. Add 100µl of staurosporine solution to well A1 of the *inhibitor titration preparative rows*. Prepare a 1:1 serial dilution of the inhibitor as shown in Figure 7.4. Mix well after each dilution by pipetting before transferring into the next well. **Note:** Do not create bubbles while preparing the diution series.

4. **Preparation of Reaction Components, 10µM ATP example:** For each kinase prepare 200µl of 2.5X ATP/Substrate Mix as described in the tables below.

### Substrate Mix Preparation

Component	Amount
4X Kinase Buffer	50µl
100µM ATP (10X)	50µl
Substrate (1mg/ml)	100µl

### Substrate Mix Preparation: If the substrate is MBP, Casein or Histone H1:

Component	Amount
4X Kinase Buffer	50µl
100µM ATP (10X)	50µl
Water	50µl
Substrate (1mg/ml)	50µl

5. Transfer 14µl of 2.5X ATP/Substrate Mix to odd numbered wells (1,3,5...23) of a 384-well plate in **Row X**. This is your *ATP/Substrate preparative row*.

6. Prepare 140µl of kinase solution (excess amount of 70 reactions at 2µl/reaction/well) as described in the table below. this will give SB10ng of kinase/reaction.

### Kinase Solution Preparation

Component	Volume
Water	Yµl = 105µl-X
4X Kinase Buffer	35µl
Kinase (100ng/µl)	Xµl = (70 × SB10/100)

7. Add 12µl of the kinase solution to odd numbered wells (1,3,5...21) and 8µl to well 23 of a 384-well plate **Row Y**, as a *kinase preparative row*.

8. **Kinase Reaction Experiment:** Transfer 2µl kinase samples in duplicate from the wells of the *kinase preparative row* to wells A1 through B22 of a 384-well plate. **Note:** Add only 2µl of 1X Kinase Buffer to wells B23-B24 for the no-enzyme control.

9. Transfer 1µl inhibitor samples in duplicate from the wells of the *inhibitor titration preparative rows* to the corresponding wells of the *assay rows* (Well A1 from the 96-well plate to well A1 and A2 of the 384-well plate, etc.)

10. Mix and incubate at room temperature for 10 minutes.

11. Transfer 2µl of the corresponding 2.5X ATP/Substrate Mix from the wells of the *ATP/Substrate preparative row* to the same *assay rows* where the kinase/inhibitor mixes are present.

12. Spin the plate. Mix for 2 minutes and then incubate the kinase reaction at room temperature for 60 minutes, or the desired time.

Well #	Final Inhibitor Concentration	Starting Volume of Each Well	Volume to Transfer
A1	10,000nM	100µl	50µl
A2	5,000nM	50µl	50µl
A3	2,500nM	50µl	50µl
A4	1,250nM	50µl	50µl
A5	625nM	50µl	50µl
A6	312.5nM	50µl	50µl
A7	156.3nM	50µl	50µl
A8	78.1nM	50µl	50µl
A9	39.1nM	50µl	50µl
A10	19.5nM	50µl	50µl
A11	9.8nM	50µl	50µl (transfer to B1)
A12	0	50µl	Buffer Only
B1	4.88nM	50µl	50µl
B2	2.44nM	50µl	50µl
B3	1.22nM	50µl	50µl
B4	0.61nM	50µl	50µl
B5	0.31nM	50µl	50µl
B6	0.15Nm	50µl	50µl
B7	0.08nM	50µl	50µl
B8	0.04nM	50µl	50µl
B9	0.02nM	50µl	50µl
B10	0.01nM	50µl	0µl
B11	0	50µl	Buffer Only
B12	No enzyme	50µl	Buffer Only

Figure 7.4. Performing serial 1:1 dilutions of inhibitor.

- ADP detection with ADP-Glo™ Kinase Assay:** After the kinase reaction incubation, add 5µl of ADP-Glo™ Reagent to all wells in your assay plate. Mix for 2 minutes and incubate the reaction at room temperature for 40 minutes.
- Add 10µl of Kinase Detection Reagent to all the wells in your assay plate. Mix for 2 minutes and incubate the reaction at room temperature for 30–60 minutes.
- Measure the luminescence (integration time 0.5 second).
- Calculating Percent Enzyme Activity:** First subtract the signal of the negative control (no enzyme and no staurosporine) from all the samples signal. Then use the 0% kinase activity (neither compound nor enzyme) and the 100% kinase activity (no compound) to calculate the other percent enzyme activities remaining in the presence of the different dilutions of staurosporine.

### Additional Resources for ADP-Glo™ Kinase and ADP-Glo™ Max Assays

#### Technical Bulletins and Manuals

- TM313 [ADP-Glo™ Kinase Assay Technical Manual](#)
- TM343 [ADP-Glo™ Max Assay](#)

#### Promega Publications

[Protocol for Kinase Inhibitor Dose Response Curve Screening and Profiling Kinase Inhibitors with a Luminescent ADP Detection Platform](#)

#### Citations

Tai, A.W. *et al.* (2011) A homogeneous and nonisotopic assay for phosphatidylinositol 4-kinases *Anal. Biochem.* **417**, 97–102.

The authors of this study evaluated the ADP-Glo™ Assay technology for use in high-throughput screening applications for inhibitors of all four known mammalian PI 4-kinases. They found that  $K_m$  values,  $IC_{50}$  values of known inhibitors, and dose-response curves were

comparable to values reported in the literature or those obtained using the standard isotopic assay.  $Z'$ -factor values for the assay in a low-volume, 384-well format were 0.72 and 0.74, indicating that the assay would be suitable for screening activities in 384- or 1536-well formats.

**PubMed Number:** 21704602

Balzano, D. *et al.* (2011) A general framework for inhibitor resistance in protein kinases *Chemistry and Biology* **19**, 966–75.

The authors of this paper investigated mutations affecting the hinge loop of protein kinases that appear to confer resistance to both Type I and Type II inhibitors. They introduced individual amino acid substitutions into the hinge region of six distantly related protein kinases and determined the inhibitor sensitivity of these kinases. The ADP-Glo™ Kinase Assay was used to assess the activity of the Haspin and c-Src kinases and the engineered mutants in this study.

**PubMed Number:** 21867912

Ohana, R.F. *et al.* (2010) HaloTag-based purification of functional human kinases from mammalian cells *Protein Expression and Purification* **76**, 154–64.

The authors of this paper demonstrate the utility of the HaloTag® protein purification system for purifying functional proteins from mammalian cells. To this end five kinases were cloned into HaloTag® vectors, expressed in and purified from HEK293T cells. To demonstrate functionality of the purified recombinant kinases, activity was measured using the ADP-Glo™ Kinase Assay.

**PubMed Number:** 21129486

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