# User Manual

## Kinase Enzyme Activity Assay Kit

For the evaluation of kinase activity and inhibition through fluorometric measurement of ADP accumulation

Document Number 70-452 Revision 0

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## Overview

The Kinase Enzyme Activity Assay is a biochemical assay for evaluating kinase activity in the screening and profiling of kinase inhibitors through the measurement of ADP accumulation. The assay kit includes all the required reagents for running the assay, including the kinase, substrate, ATP, detection reagents, and assay plates. Each target and lot-specific accompanying Certificate of Analysis (COA) provide the details of the optimal reaction conditions for the detection of kinase activity. Depending on the kit size purchased, the kit includes sufficient material to run reactions for either 2, 6, or 25 384-well assay plates.

## Assay Principle

Eurofins DiscoverX® Kinase Activity Assay kits utilize the ADP Hunter™ Plus Assay platform. ADP Hunter Plus is a homogeneous assay for measuring ADP accumulation, a universal byproduct of kinase enzyme activity, in a convenient gain-of-signal fluorometric assay format in 90 minutes. ADP Hunter Plus assay platform is a coupled enzyme reaction system that generates hydrogen peroxide from accumulated ADP resulting from the kinase enzyme reaction. Hydrogen peroxide, when combined with ADHP (fluorescent dye precursor) in the presence of peroxidase, generates the quantitative resorufin-based signal that can be read at 590nm. To allow for automation, a Stop Solution is also provided for added signal and background stabilization (see Figure 1 below).

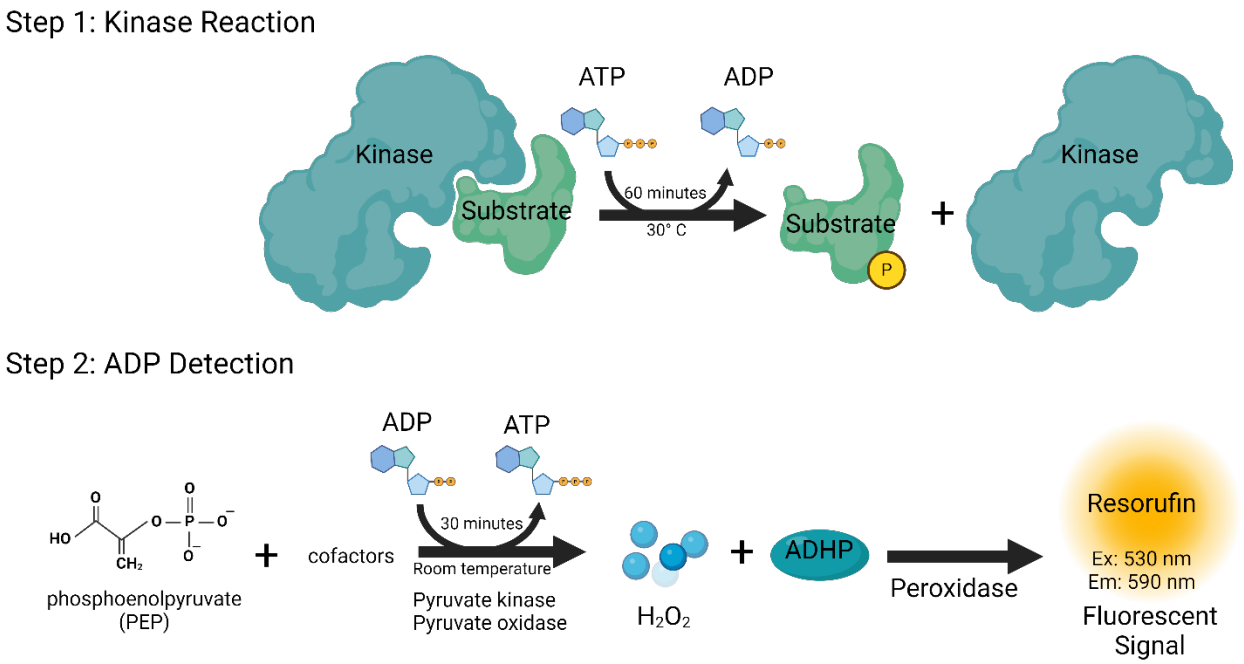


**Figure** 1**. Kinase Enzyme Activity Assay Principle**

## Assay Workflow

Please read the entire protocol completely before running the assay. Successful results depend on performing these steps correctly. The Assay Procedure sections, the Kinase Enzyme Activity Assay Kit Certificate of Analysis (CoA), and the Quick Start Guide contain detailed information about how to run the assays.

### Inhibitor Determination

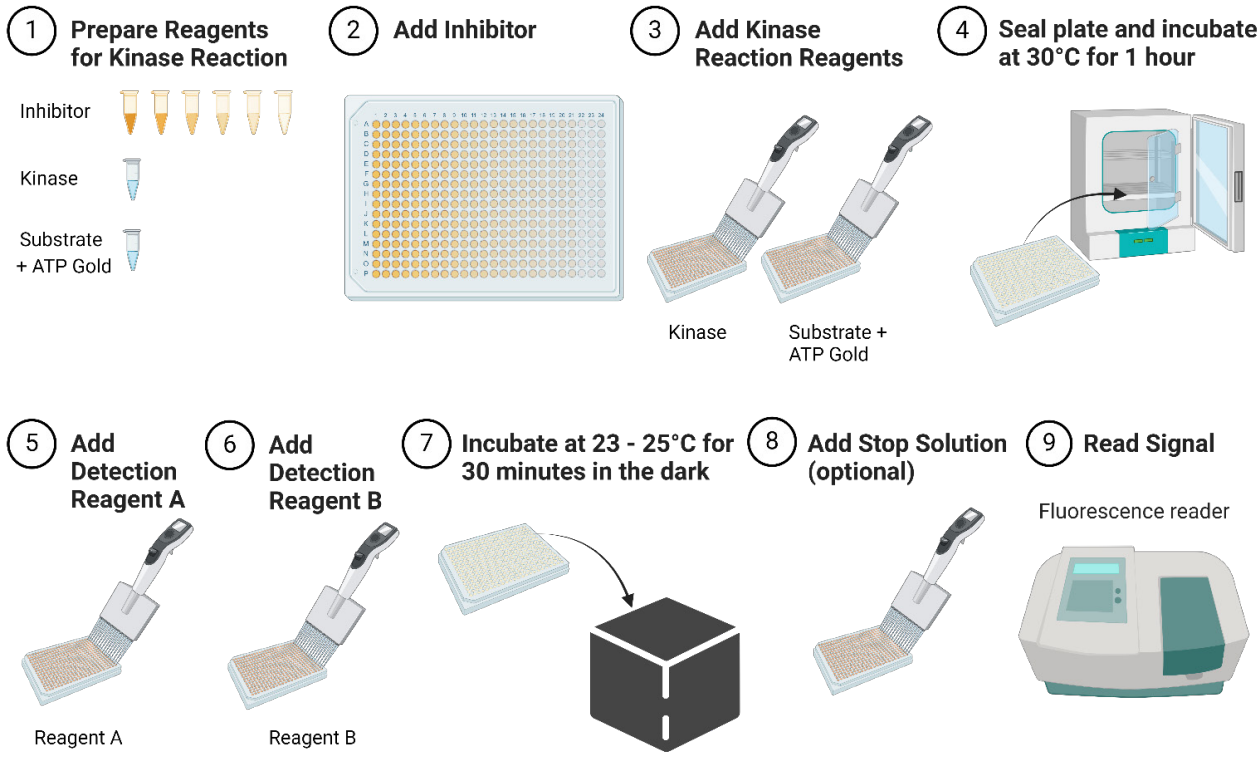


**Figure 2. Kinase Reaction Inhibitor Determination Assay Workflow**

Screen and profile kinase inhibitors directly through measurement of ADP accumulation by performing the steps listed above (Figure 2). For reagent volumes and preparation instructions, refer to the "Detailed Assay Protocol" section of this manual.

## ADP Standard Curve

This section describes the assay workflows for generating an ADP standard curve and performing a kinase reaction procedure for inhibitor determination.

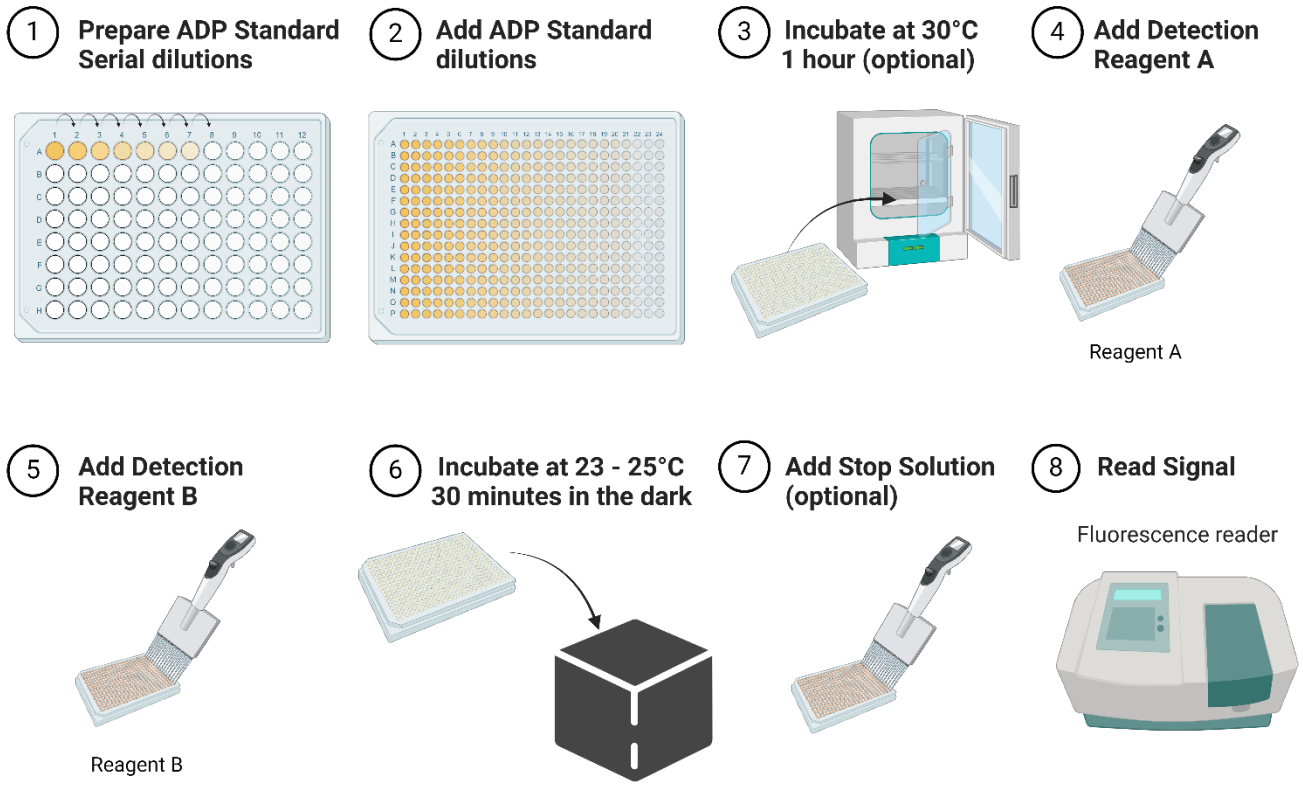


**Figure 3. ADP Standard Curve Assay Workflow**

Figure 3 illustrates the assay steps required for the generation of the ADP standard curve. For reagent volumes and preparation instructions, refer to the “Detailed Assay Protocol” section of this manual.

## Materials Provided

| List of Components | 2-Plate Kit | 6-Plate Kit | 25-Plate Kit |
|---|---|---|---|
| Kinase (refer to the specific CoA for details) | See CoA | See CoA | See CoA |
| Substrate (refer to the specific CoA for details) | See CoA | See CoA | See CoA |
| ATP Gold (25 mM) | See CoA | See CoA | See CoA |
| ADP Hunter™ Plus Assay Kit: - Reagent A[1] | 8 mL | 3 x 8 mL | 100 mL |
| - Reagent B[1] | 16 mL | 3 x 16 mL | 200 mL |
| - Stop Solution | 4 mL | 3 x 4 mL | 50 mL |
| - ADP Standard (225 µM) | 2 mL | 3 x 2 mL | 25 mL |
| - Assay Buffer | 50 mL | 3 x 50 mL | 625 mL |
| 384-well Non-Binding Black, FB | 2 plates | 6 plates | 25 plates |
| Top Seal | 2 seals | 6 seals | 25 seals |

[1] Reagents A and B may show some particulate matter upon thawing. This does not affect the performance of the assay.

**Table 1. Materials Provided**

**Note:** The ADP Hunter™ Plus Assay Buffer is used to prepare the substrate, the kinase, and ATP Gold. The assay buffer is pH 7.4 containing 15 mM HEPES, 20 mM NaCl, 1 mM EGTA, 0.02% Tween 20, 10 mM $MgCl_2$, and 0.1 mg/mL BGG (bovine-γ-globulins).

## Storage and Stability

The Kinase Enzyme Activity Assay Kit is stable until the expiration date indicated on the box's outer label when stored at the recommended storage temperatures indicated for kit components in this section. For maximum product recovery, centrifuge the original vial prior to removing the cap.

### Kinase and Substrate

Upon receipt, store kinase and substrate at -70°C.

### ATP Gold and ADP Hunter™ Plus kit

Upon receipt, store ATP Gold and the ADP Hunter™ Plus kit reagents at the recommended temperature of -20°C or -70°C. Aliquots of the kit components should be made upon initial thaw and stored at -20°C or -70°C. Reagents can be thawed up to 3 times with no effect on performance.

### 384-well Non-Binding Black, FB plates, and Top Seal

Store at room temperature.

## Handling Recommendations

Kinase and substrate: Thaw the vials on ice. Reagents can be thawed up to 3 times with no effect on performance. For unused material, snap freeze vials on dry ice prior to re-storage at -70°C.

ADP Hunter™ Plus Kit and ATP Gold: Equilibrate reagents to ambient room temperature before use.

## Additional Materials Required

The following materials are required.

| Equipment | Materials |
|---|---|
| Fluorescence intensity reader | Pipettes and pipette tips |
| Filter wavelengths required for Resorufin:<br>Peak Excitation wavelength: 530 nm<br>Peak Emission wavelength: 590 nm | 96-well plate for compound dilution |

**Table 2. Materials Required**

## Guidelines for Use

The assay consists of three steps: step 1 is the kinase reaction, step 2 is the addition of the detection reagents, and step 3 (optional) is the addition of the assay stop solution.

**Kinase Reaction:** The kinase reaction includes a purified active kinase, a substrate (peptide, protein, or lipid), ATP, and compounds, all in an appropriate kinase reaction buffer.

See optimized concentrations and volumes in kit CoA for kinase, substrate, and ATP gold preparation.

**ADP Hunter Plus Reagent A:** Ready to use, no preparation needed.

**ADP Hunter Plus Reagent B:** Ready to use, no preparation needed.

**ADP Hunter Plus Stop Solution:** Ready to use, no preparation needed.

**Controls:** A control without kinase should be included in each experiment. The signal from this control may be used to correct the background signal. This control should contain ATP, substrate, and compound or vehicle.

### Tips for optimal performance:

- ADP Reagent A and Reagent B must be added separately. Do not pre-mix.
- Reagent A must be added before Reagent B for optimal assay performance.
- If needed, Stop Solution must be added last after the 30-minute incubation of Reagent A and B. Do not combine with either Reagent A or Reagent B.
- The Stop Solution stabilizes the signal and background.

## Protocol Schematic

**Tip:** *Use this sheet to note your assay specific conditions. Post it on your bench to use as a quick reference guide.*

Assay Name: _____ Date: _____

Product Details: _____

Quick-start Procedure: In a 384-well microtiter plate, perform the following steps.

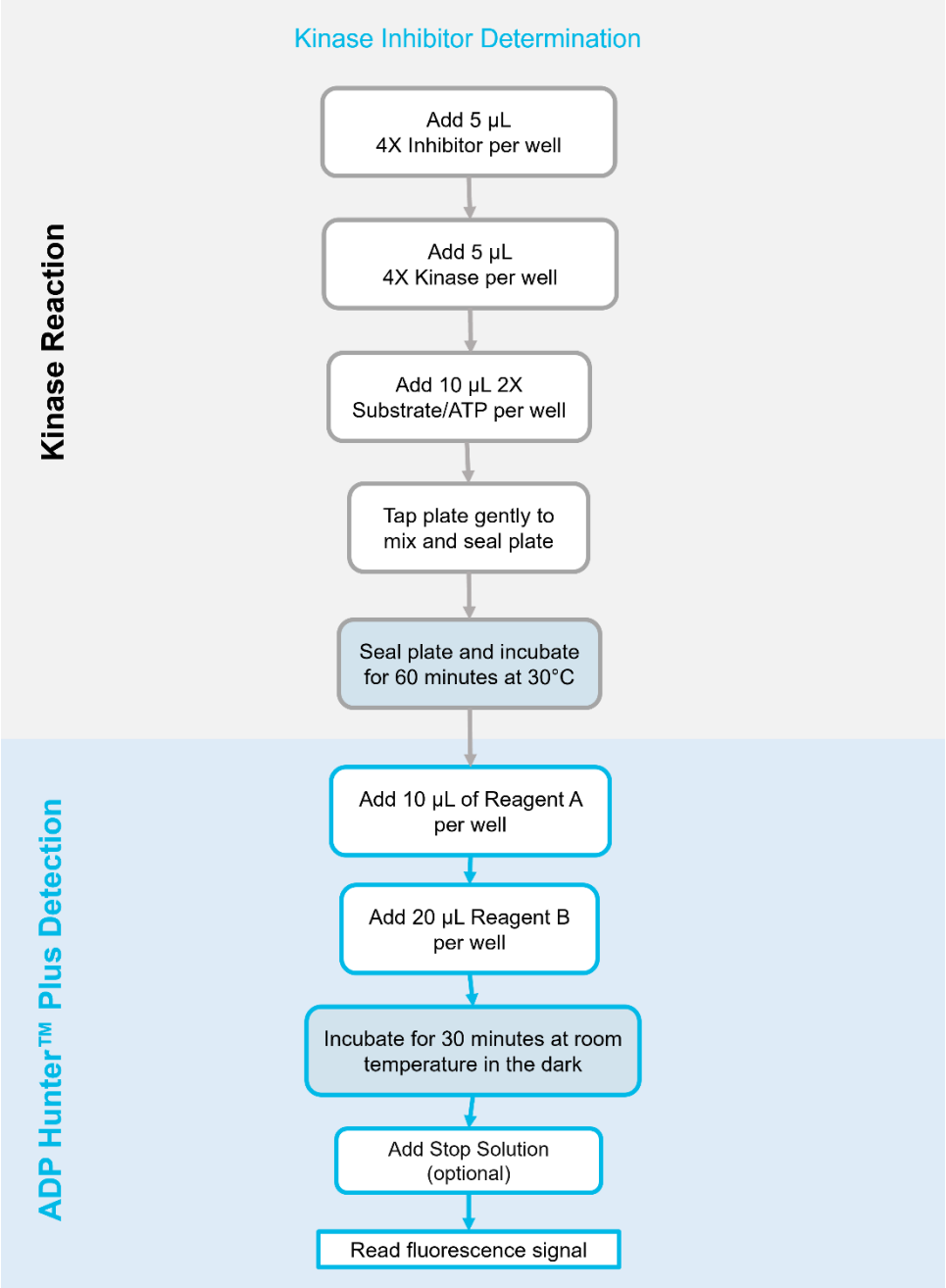


**Figure 4. Quick-start Procedure**

# Detailed Assay Protocol (Standard Curve)

## ADP Standard Preparation

Prepare 3-fold serial dilutions of the ADP Standard in the Assay Buffer provided in the kit. Add 100 µL of Assay Buffer per well in 8 wells of a 96-well plate. Add 50 µL of the Standard to the first well. Then transfer 50 µL to the next well. Serially dilute 5 more times and do not add any diluted Standard to the last well. Use the assay buffer in the last well as the zero ADP control. The ADP Standard concentrations range from 0.1 µM to 75 µM in the kinase reaction.

**Note:** 40 µL per concentration is required for one standard curve (7 points curve plus zero control, duplicate replicas per concentration).

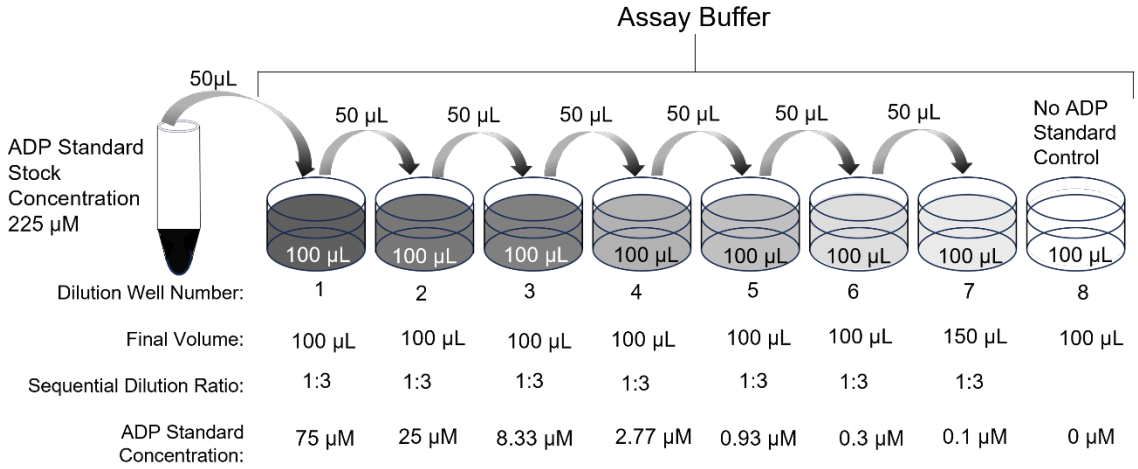


**Figure 5. ADP Standard Serial Dilution**

## ADP Standard Addition, Detection, and Plate Reading

The Assay Overview table below (Table 3) outlines the per well reagent volumes and procedure for the ADP standard curve for a full volume 384-well plate format.

## Assay Overview for 384-well plate with reagent volumes per well

| Assay Step | ADP Standard Curve | Inhibitor Determination |
|---|---|---|
| **Step 1: Standard Dilutions / Kinase Reaction** | 20 µL ADP Standard dilutions | 5 µL of inhibitor preparation<br>5 µL of Kinase preparation<br>10 µL of Substrate and ATP Gold preparation |
| **Incubate for 1 hour at 30°C[1]** | | |
| **Step 2: ADP Detection** | Add 10 µL Reagent A<br>Then<br>Add 20 µL Reagent B | Add 10 µL Reagent A<br>Then<br>Add 20 µL Reagent B |
| **Incubate for 30 minutes at room temperature (protect from light)** | | |
| **Step 3: Stop Solution (optional)** | Add 5 µL Stop Solution | Add 5 µL Stop Solution |
| **Read Fluorescence Intensity signal:<br>Resorufin Excitation wavelength – 530 nm<br>Resorufin Emission wavelength – 590 nm** | | |
| **Note: The signal may be measured up to 1 hour after the addition of the Stop Solution.<br>The addition of the Stop Solution will result in a 10% reduction in the assay window.** | | |

[1] Optional incubation step if the assay plate is used exclusively for ADP Standard Curve data generation.

**Table 3. Step-by-step overview: ADP Standard Curve / Kinase Reaction for Inhibitor Determination**

## Detailed Assay Protocol (Kinase Reaction Procedure for Inhibitor Determination)

Inhibitor compounds, Kinase, Substrate, and ATP Gold must be diluted in the ADP Hunter™ Plus Assay Buffer. The kit CoA provides the recommended concentrations to use for a successful assay. Inhibitor compounds are prepared at a 4X concentration and 5 µL added per well. The kinase is prepared at a 4X concentration and 5 µL added per well. A master mix of the Substrate and ATP is prepared at 2X of the final assay concentration and added at 10 µL per well to initiate the kinase reaction. The total kinase reaction volume is 20 µL.

### Assay Overview

The Assay Overview table above (Table 3) summarizes the assay volumes and procedure for a full-volume 384-well format. The detailed protocol above includes reagent addition volumes for the reaction, detection, and optional stop solution steps. Run appropriate controls with all experiments.

### Inhibitor Master Mix Preparation and Addition

1. Prepare a 4X master mix of inhibitor in ADP Hunter Plus Assay Buffer
   - For single point determination, 10 µM is a typical test concentration. Include several wells for the vehicle control as this defines activity in the absence of kinase inhibition.
2. Add 5 µL 4X the inhibitor master mix just prepared into each test well of the assay plate.
3. Cover the plate to avoid evaporation and proceed to the next step.

### Kinase Master Mix Preparation and Addition

1. Prepare a 4X kinase master mix in ADP Hunter Plus Assay Buffer.

| Reagent | 4X master mix concentration |
|---|---|
| Kinase | See the concentration in the relevant table included in the kit specific CoA. |

**Table 4. Kinase preparation**

2. Add 5 µL of the 4X kinase master mix to all wells containing inhibitors or vehicle controls.
3. After the kinase master mix is added to each test well, the plate can be tapped gently to mix with the compound.
4. Cover the plate to avoid evaporation and proceed to the next step.

### Substrate and ATP Gold Master Mix Preparation and Addition

1. Prepare a 2X Substrate and ATP Gold master mix in ADP Hunter Plus Assay Buffer.

| Reagent | 2X master mix concentration |
|---|---|
| Substrate | See the concentration in the relevant table included in the specific kit CoA. |
| ATP Gold | See the concentration in the relevant table included in the specific kit CoA. |

**Table 5. Substrate and ATP Gold Preparation**

2. Add 10 µL of the 2X Substrate/ATP master mix just prepared into each well of the assay plate.
3. After the master mix is added, the plate can be tapped gently to mix the kinase reaction.
4. Seal the plate with the provided plate seal.
5. Incubate the plate for 60 minutes at 30°C.

**Note:** The reaction is initiated with the addition of ATP.

### Assay Detection and Plate Reading

1. Optional—Add 5 µL the Stop Solution to each assay well for use in settings with automation. Note that the addition of the Stop Solution will result in a 10% reduction in the assay window. The signal may be measured up to 1 hour after the addition of the Stop Solution.
2. Read the fluorescence intensity signal with an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

## Document Revision History

| Revision Number | Date Released | Revision Details |
|---|---|---|
| 0 | May 2024 | Initial version of the Kinase Enzyme Activity Assay User Manual |

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