Technical Bulletin

Kinase Assay Kit

Catalog Number MAK441

Product Description

Kinases, also known as phosphotransferases, constitute a large family of enzymes that transfer phosphate groups from the high-energy donor molecule ATP to their specific substrates. Kinases are known to regulate the majority of cellular processes. The largest group of this family is the protein kinases. So far, 518 different kinases have been identified in humans, and up to 30% of human proteins are modified by these kinases. The enormous diversity of kinases in biological systems and their key role in cellular signaling make them ideal targets for drug development.

The Kinase Assay Kit provides a simple and rapid method for assaying kinase activity and for high-throughput screening of kinase inhibitors. This microplate-based assay involves incubating the kinase with a single working reagent, in which ADP is enzymatically converted to ATP and pyruvate, which then may be quantified by measuring the resulting fluorometric signal (λ_{Ex} = 530 nm/λ_{Em} = 590 nm).

The kit is suitable for the determination of protein kinase activity and for high-throughput screening of kinase inhibitors.

Components

The kit is sufficient for 400 fluorometric assays in 384-well plates or 200 assays in 96-well plates.

- Reagent A 10 mL
  Catalog Number MAK441A
- Reagent B 10 mL
  Catalog Number MAK441B
- Assay Buffer 25 mL
  Catalog Number MAK441C
- Standard (3 mM ADP) 100 µL
  Catalog Number MAK441D

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Fluorescent multiwell plate reader
- Black flat-bottom 96-well or 384-well plates. Cell culture or tissue culture treated plates are **not** recommended.
- Adenosine 5′-triphosphate disodium salt hydrate (ATP)
  (Catalog Number A7699 or equivalent)
- Appropriate substrate for use with the kinase of interest. The substrate must be selected based on the kinase to be studied and may be a protein or synthetic peptide.

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C.

Preparation Instructions

Briefly centrifuge small vials prior to opening. Equilibrate all components to room temperature prior to use.

Note: Thiols such as β-mercaptoethanol, dithioerythritol, etc., at concentrations >10 µM interfere with this assay and should be avoided.

Procedure

All samples and standards should be run in duplicate.

Note: This procedure is written for 384-well plates. if utilizing 96-well plates, multiply all volumes to be used by a factor of 4.

Kinase Reaction Mixture (384-well format)

Note: Users must provide their own kinase enzyme (Sample), ultra-pure ATP (e.g., Catalog Number A7699) and substrate.

1. Into a black 384-well plate, prepare a 20 µL reaction mixture per well containing the kinase, ATP and substrate in the provided Assay Buffer (pH 7.0) or any suitable kinase buffer. Typical kinase assays use 100 to 200 µM ATP.

   Note: The user must optimize assay conditions for their kinase of interest and substrate. The use of 10 µL or more of Assay Buffer per 20 µL reaction volume is recommended. Suggested reagent volumes per reaction are 0.5 – 2 µL ATP and 0.5 – 2 µL Substrate.
2. Prepare a 20 µL Blank Control that contains ATP and substrate but no kinase enzyme in separate wells of the plate. Replace the same volume of kinase enzyme with Assay Buffer.
3. Incubate at desired temperature for desired period of time (e.g., 30 minutes).

Standard Curve Preparation

1. Prepare a 10 µM ADP Standard by mixing 3 µL of the 3 mM ADP Standard with 897 µL of purified water.
2. Prepare Acetylcholine Standards in 1.5 mL microcentrifuge tubes according to Table 1.

Table 1.
Preparation of ADP Standards

Well	10 µM ADP Standard	Purified Water	ADP (µM)
1	100 µL	-	10
2	60 µL	40 µL	6
3	30 µL	70 µL	3
4	-	100 µL	0

3. Mix well and transfer 20 µL of each Standard into separate wells of a black 384-well plate.

ADP Detection Working Reagent

Mix enough reagents for the number of assays to be performed. For each Standard and Sample well, prepare 50 µL of Working Reagent according to Table 2.

Table 2.
Preparation of Working Reagent

Reagent	Working Reagent
Reagent A	25 µL
Reagent B	25 µL

Measurement

1. Add 40 µL of Working Reagent to each Sample and Standard well.

2. Tap plate to mix and incubate for 10 minutes at room temperature.
3. Measure the fluorescence intensity (F) at λ_{Ex} = 530 nm/λ_{Em} = 590 nm.

Results

1. Subtract the Blank (0 Standard) (F) reading from all Standard (F) readings.
2. Plot the corrected Standard readings (ΔF) against the Standard concentrations and determine the Standard Curve.
3. Calculate the corrected fluorescence reading of the Sample (ΔF_S) by subtracting the Blank Control (F) reading from each Sample (F) reading.
4. Typical kinase assays use 100 to 200 µM ATP. If the ΔF of the Sample > ΔF of the 10µM Standard, dilute the enzyme in assay buffer. Repeat the assay and multiply the results by the dilution factor (DF). This will ensure that the initial rate is measured at < 10% ATP conversion.
5. Calculate the kinase activity of Sample.

Kinase (U/L) =

$$\frac{\Delta F_S}{Slope \times T} \times \frac{20}{V}$$

where

ΔF_S = Fluorescence intensity of the Sample well – Fluorescence intensity of the Blank Control well

Slope = Slope of the ADP Standard curve

T = Kinase reaction time (e.g., 30 minutes)

20 = Kinase Reaction Mixture volume (µL)

Vol = Volume (in µL) of Sample Kinase enzyme added to the Kinase Reaction Mixture

<u>High-throughput Screen for Kinase Inhibitors (384-well format)</u>

For inhibitor screens, Test Compounds are usually pre-incubated with kinase for 10 to 30 minutes prior to adding ATP/substrate to initiate the kinase reaction. After a 30 minute enzymatic kinase reaction, the produced ADP is quantified using the fluorometric assay. The fluorescence intensity (F) will be decreased in the presence of an inhibitor.

1. <u>Controls and Test Compounds:</u> Use a known kinase inhibitor (e.g., staurosporine) as a positive control. Alternatively, "no enzyme" wells can serve as a positive control. Use the same volume of the Test Compound solvent (e.g., DMSO) as an inhibitor negative control. For example, transfer 5 µL of the Test Compound in DMSO, Control Inhibitor, and Negative Control DMSO to appropriate wells. Add 10 µL of kinase enzyme solution to all assay wells. Apply an "in-well" mixing step. Incubate for 10 to 30 minutes.
2. <u>Kinase Reaction:</u> Add 5 µL of a Reaction Mixture containing ATP and the kinase substrate to each assay well. Mix well. Incubate for desired period of time (e.g., 30 minutes).
3. <u>ADP Detection:</u> Prepare enough Working Reagent for each well by mixing 25 µL of Reagent A and 25 µL of Reagent B. Add 40 µL Working Reagent to each well and mix well. Incubate for 10 minutes.
4. <u>Measurement:</u> Read fluorescence intensity at λ_{Ex} = 530 nm/λ_{Em} = 590 nm.

Figure 1.
Enzyme titration with AMPK

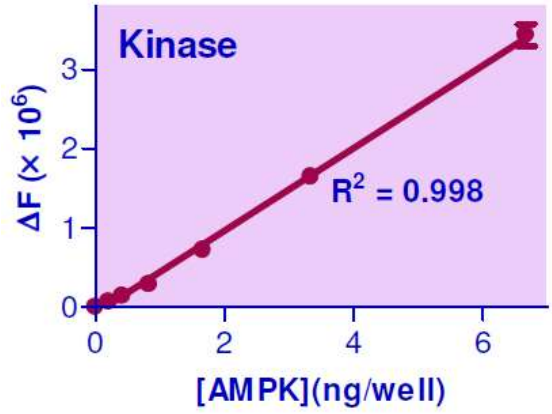
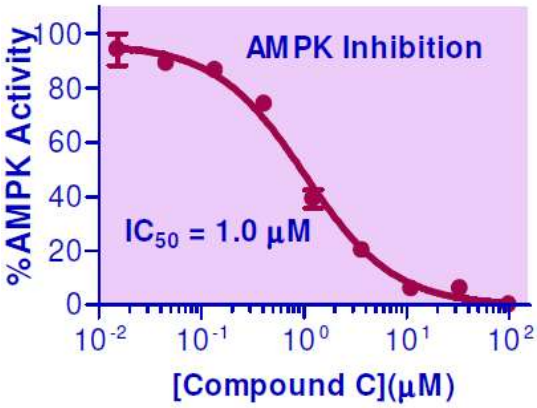


Figure 2.
Inhibition curve with 2 ng AMPK and 50 µM ATP



References

1. Liu, K., et al., Different p53 genotypes regulating different phosphorylation sites and subcellular location of CDC25C associated with the formation of polyploid giant cancer cells. *J. Exp. Clin. Cancer Res*., **39**, 83 (2020).
2. Hirooka, K., et al., Regulation of the rhaEWRBMA operon involved in L-rhamnose catabolism through two transcriptional factors, RhaR and CcpA, in *Bacillus subtilis*. *J. Bacteriol.*, **198**, 830-845 (2016).

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