Phos-tag-based non-radioactive protocols for monitoring Arabidopsis kinase activities in vitro

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Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Phos-tag-based non-radioactive protocols for monitoring Arabidopsis kinase activities in vitro

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SUMMARY
Kinases are indispensable signaling components. Radioactive-based phosphorylation assays are widely used but require specific protective equipment and safety trainings. Here, we present a Phos-tag-based non-radioactive kinase assay to study Arabidopsis kinase activities. We expressed and purified both kinase and substrate proteins from E. coli cells and then used the Phos-tag technology to detect the kinase activities under either different temperatures or chemical treatments. This non-radioactive approach is environmentally friendly and applicable to other kinases and organisms.

BEFORE YOU BEGIN
Kinase reactions are indispensable for signal transduction, energy production, metabolism, and defense responses in both plants and animals. Kinase activities are sensitive to temperature variations. For example, low temperature activates OST1 kinase to enhance freezing tolerance in plants (Ding et al., 2015). High temperature inhibits the activity of LAMMER kinase CLK1/4 in mice and human and thus reduces the phosphorylation levels of their substrates serine/arginine-rich (SR) proteins (Haltenhof et al., 2020). We recently reported that high temperature also repressed the Arabidopsis CLK kinase homolog AFC2 activities (Lin et al., 2022). On another side, given the crucial roles of kinase, screening small molecules to target specific kinase is promising in pharmaceutical drug development for disease treatment (Overington et al., 2006; Mifflin et al., 2020; Attwood et al., 2021). To determine kinase activities, radioactive based phosphorylation assays are the most popular approaches in the past decades (Ding et al., 2015; Haltenhof et al., 2020). However, these assays are not environmentally-friendly and cannot be scaled up due to the limitations from specific safety requirements. Although there are also numerous other approaches to determine kinase activities in a non-radioactive way, such as fluorescence resonance energy transfer (FRET)-based kinase assays, these methods require expensive specific machine for signal detections. Here, we described a Phos-tag gel based non-radioactive protocol for measuring Arabidopsis kinase activities (kinase: AFC2/At4g24740; substrate: RSZ21/At1g23860) under different temperatures or inhibitor concentrations. Our protocol is simple and easy to be optimized for the detection of kinase activities from any other organisms.

Constructs and protein expression trials

© Timing: 3 days

1. The full-length coding sequence of AFC2 was cloned into the glutathione s-transferase (GST) fusion protein expression vector pGEX-5x-1 (ampicillin resistance) and transformed into E. coli BL21 (DE3) competent cells.
2. The full-length coding sequence of RSZ21 was cloned into maltose binding protein (MBP) fusion protein expression vector pMAL-c5x (ampicillin resistance) and transformed into E. coli BL21 (DE3) competent cells.

⚠ CRITICAL: Make sure the coding sequence inserted into the vector is correctly constructed in frame with the tag.

**Alternatives:** It is recommended to use different tags for the kinase and substrate fusions, respectively. These commercial expression vectors also contain different epitope tag locations, you may choose N-terminus or C-terminus fusion types (depending on the expression and purification efficiency). But if you have specific antibodies to detect both kinase and substrate, you can use those antibodies in the western blot. At this circumstance, you can express both proteins with the same tag and purify them simultaneously.

**Alternatives:** The E. coli strain used in this experiment is BL21 (DE3). You may try other modified strains, such as Rosetta (DE3) for obtaining high expression efficiency.

3. Test protein induction conditions.
   a. Streak a single transformed E. coli colony into 5 mL of LB liquid medium supplemented with 50 µg/mL ampicillin in a 15 mL centrifuge tube (all the plastic products used in this protocol are sterile, if not specified), and incubate at 37°C shaker for 12–14 h at 230 rpm.
   b. Prepare seven 15 mL centrifuge tubes with 4.3 mL of LB liquid medium supplemented with 50 µg/mL ampicillin each, then add 700 µL of bacterial culture into each centrifuge tube, and incubate at 37°C shaker to OD$_{600}$ = 0.6–0.8 at 230 rpm.

**Note:** Label one 15 mL centrifuge tube as the non-induction control and the other six 15 mL centrifuge tube for screening the best protein induction condition.

c. Transfer 1 mL of bacterial culture from the control group into a 1.5 mL microcentrifuge tube (Eppendorf tube) as a non-induction control.

d. Centrifuge the non-induction control Eppendorf tube for 1 min at 13,523 g and discard the supernatant.

e. Add 30 µL of 4 × SDS loading buffer to the pellet without mix. Immediately boil the Eppendorf tube for 10 min at 100°C.

**Note:** The non-induction control Eppendorf tube can be stored at −80°C temporarily.

f. We set six trials for screening the best protein induction condition. Apply different isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations and/or incubation temperatures in Eppendorf tube 1–6.

| Eppendorf tube number | IPTG concentration | Incubation temperature |
|-----------------------|--------------------|------------------------|
| 1                     | 1 mM               | 37°C                   |
| 2                     | 0.7 mM             | 37°C                   |
| 3                     | 0.3 mM             | 37°C                   |
| 4                     | 1 mM               | 30°C                   |
| 5                     | 0.7 mM             | 30°C                   |
| 6                     | 0.3 mM             | 30°C                   |

g. Incubate the six 15 mL centrifuge tubes for 3 h at 230 rpm.

**Note:** The protein induction condition described here is optimized for our cases. You may change the parameters and do more trials for obtaining the higher induction efficiency for
your protein-of-interest. For example, the incubation temperature could be optimized. Sometimes lower temperature (16°C) results in higher protein expression results. Meanwhile, the IPTG concentrations could be scaled up from 0.1 mM to 1 mM.

△ CRITICAL: We hope to obtain enough soluble proteins for further kinase assays. However, it is not uncommon to come across inclusion bodies during protein purification.Expressed proteins may aggregate when IPTG concentrations are high or temperature is not suitable. Therefore, a series of IPTG concentrations and incubation temperatures are required for trials.

h. Transfer 1 mL of bacterial culture from each 15 mL centrifuge tube into a 1.5 mL Eppendorf tube.
i. Centrifuge Eppendorf tubes for 1 min at 13,523 g and discard the supernatant.
j. Add 30 μL of 4 × SDS loading buffer to the pellets without mix. Immediately boil the Eppendorf tubes for 10 min at 100°C.
k. Centrifuge the non-induction control Eppendorf tube and the six Eppendorf tubes for 1 min at 4°C at 13,523 g.
l. Perform routine SDS-PAGE electrophoresis.
m. Stain the gel with Coomassie brilliant blue G-250 staining solution for 1–2 h and decolorizing the gel with decolored solution overnight (8–12 h). Check out the protein expression results and select the best condition for large scale protein expressions.

△ CRITICAL: Try to avoid mixing the pellets with 4 × SDS loading buffer, just add loading buffer to the Eppendorf tube containing the pellets, and the total protein would be released to the loading buffer after boiling. Otherwise, mixing the pellets and loading buffer would cause the loading buffer sticky (DNA release and lots of bacterial debris) and difficult for gel loading.

Pause point: SDS loading buffer treated samples can be stored at −80°C for a short while.

Note: It is better to perform SDS-PAGE electrophoresis immediately after protein extractions, make sure SDS-PAGE gels are already stacked.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| GST-Tag Antibody (1:5000 dilution) | Affinity Biosciences | Cat# T0007; RRID: AB_2839414 |
| Anti-MBP Monoclonal Antibody (1:10000 dilution) | New England Biolabs | Cat# E8032S; RRID: AB_1559730 |
| Bacterial and virus strains |        |            |
| BL21 (DE3) competent cell | Vazyme | Cat# CS04-02 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Triton X-100        | Sigma-Aldrich | Cat# V900502 |
| Phos-tag Acrylamide | Wako | Cat# 304-93521 |
| WIDE-VIEW Prestained Protein Size Marker III | Wako | Cat# 230-02461 |
| 180 kDa Prestained Protein Marker | Vazyme | Cat# MP102 |
| Manganese (II) chloride tetrahydrate (MnCl₂) | Sigma-Aldrich | Cat# V900197-500G |
| Sodium dodecyl sulfate | Sigma-Aldrich | Cat# V900859-500G |
| Ammonium persulfate substitute (APS substitute) | Beyotime | Cat# ST005-10g |
| Glycine             | Sigma-Aldrich | Cat# V900144-5KG |
| Methanol            | Sinopharm | Cat# 10014118 |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma-Aldrich | Cat# V900106-500G |

(Continued on next page)
## MATERIALS AND EQUIPMENT

### 1.5 M Tris buffer pH 8.8

| Reagent                              | Final concentration | Amount       |
|--------------------------------------|---------------------|--------------|
| Trizma base                          | 1.5 M               | 18.17 g      |
| 10% SDS                              | 0.4%                | 4 mL         |
| HCl                                  | N/A                 | to pH = 8.8  |
| ddH₂O                                | N/A                 | to 100 mL    |
| Total                                | N/A                 | 100 mL       |

Store at 4°C for 1 year.

### 0.5 M Tris buffer pH 6.8

| Reagent                              | Final concentration | Amount       |
|--------------------------------------|---------------------|--------------|
| Trizma base                          | 0.5 M               | 6.06 g       |
| 10% SDS                              | 0.4%                | 4 mL         |

*(Continued on next page)*

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**Protocol**

**Continued**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Glutathione Sepharose beads | Cytiva | Cat# 17075601 |
| Amylose resin | New England Biolabs | Cat# E8021S |
| Phenylmethylsulphonyl fluoride (PMSF) | Sigma-Aldrich | Cat# P7626 |
| L-Glutathione reduced | Sigma-Aldrich | Cat# V900436-25G |
| D- (+)-Maltose monohydrate | Sigma-Aldrich | Cat# V900435-100G |
| Trizma base | Sigma-Aldrich | Cat# V900483-5KG |
| Magnesium chloride hexahydrate (MgCl₂) | Sigma-Aldrich | Cat# V900020-500G |
| dithiothreitol (DTT) | Beyotime | Cat# ST040-5g |
| adenosine triphosphate (ATP) | Coolaber | Cat# CA1261-5G |
| Isopropyl-β–D- thiogalactopyranoside (IPTG) | Solarbio | Cat# I8070 |
| Coomassie brilliant blue G-250 | Solarbio | Cat# C8420 |
| 30% Acryl/Bis | Sangon Biotech | Cat# BS46017-0500 |
| N, N’, N”, N’-Tetramethyl ethylenediamine (TEMED) | Sigma-Aldrich | Cat# V900853 |
| Lysoyzme | Solarbio | Cat# L8120 |
| Tween-20 | Sigma-Aldrich | Cat# V900548 |
| TG-003 | Sigma-Aldrich | Cat# T5575 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | Cat# V900090 |
| KCI | Sinopharm | Cat# 10016318 |
| KH₂PO₄ | Sinopharm | Cat# 10017618 |
| Na₂HPO₄·12H₂O | Sinopharm | Cat# 10020318 |
| NaCl | Sinopharm | Cat# 10019318 |
| Acetic acid | GHTECH | Cat# 1.14100.058 |
| Ethanol absolute | Sinopharm | Cat# 10009527 |
| Ampicillin | Solarbio | Cat# A8180 |

**Recombinant DNA**

| GST-AFC2 | Lin et al. (2022) | N/A |
| MBP-RS221 | Lin et al. (2022) | N/A |

**Other**

| 0.22 μm PES Membrane Filter Unit | Millipore | Cat# SLGPR33RB |
| Protein purification column | Bio-Rad | Econo-Pac columns |
| Nitrocellulose membrane | Pall | Cat# P-N66485 |
| Transference Decoloring Shaker | Kylin-Bell | TS-8 |
| Eppendorf heater | Eppendorf | Thermomixer comfort |
| Spectrophotometer | DeNovix | DS-11 |
| Gel Image Analysis System | Tanon | Tanon-4100 |
### 10% SDS-PAGE separating gel

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| 1.5 M Tris buffer pH 8.8 | 0.375 M             | 1.4 mL  |
| 30% Acryl/Bis            | 10%                 | 1.848 mL|
| 10% APS                  | 0.1%                | 56 µL   |
| TEMED                    | N/A                 | 4.48 µL |
| ddH₂O                    | N/A                 | 2.296 mL|
| Total                    | N/A                 | 5.6 mL  |

Store at 4°C with humidity for up to 1 day.

### SDS-PAGE stacking gel

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| 0.5 M Tris buffer pH 6.8 | 0.125 M             | 584 µL  |
| 30% Acryl/Bis            | 5%                  | 385 µL  |
| 10% APS                  | 0.1%                | 23.5 µL |
| TEMED                    | N/A                 | 2.35 µL |
| ddH₂O                    | N/A                 | 1.34 mL |
| Total                    | N/A                 | 2.33 mL |

Store at 4°C with humidity for up to 1 day.

### 1.5 M Tris-HCl pH 8.8

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Trizma base              | 1.5 M               | 18.17 g |
| HCl                      | N/A                 | to pH = 8.8|
| ddH₂O                    | N/A                 | to 100 mL|
| Total                    | N/A                 | 100 mL  |

Store at 4°C for 1 year.

### 5 mM Phos-tag Acrylamide

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Phos-tag Acrylamide      | 5 mM                | 10 mg   |
| Methanol                 | 3%                  | 100 µL  |
| ddH₂O                    | N/A                 | 3.2 mL  |
| Total                    | N/A                 | 3.3 mL  |

After wrapping with aluminum foil, keep away from light at 4°C for 1 year.

**Note:** Phos-tag Acrylamide is completely dissolved in 0.1 mL of methanol and then add 3.2 mL of distilled water to dilute.

**CRITICAL:** It becomes opaque when adding ddH₂O and then it can be used after resting a while and becoming transparent. If there are insoluble impurities in the solution, centrifuge for 10 min at 2,000 g in a 2 mL microcentrifuge tube to remove them.
Note: Increasing Phos-tag Acrylamide concentration may improve protein band separation. However, higher concentrations of Phos-tag Acrylamide slow down the protein migration speed. Therefore, it is recommended to gradually increase the Phos-tag Acrylamide concentration from 20 μM to 100 μM to find the best electrophoresis condition. For samples with relatively very low protein concentrations, a higher Phos-tag concentration (such as 100 μM) is recommended.

Note: The molar concentration of MnCl₂ is two times of Phos-tag Acrylamide.

Note: For separating proteins with molecular weight less than 60 kDa, we recommend to use 8% gel. For proteins with molecular weight larger than 60 kDa, 6% gel is encouraged. For proteins with molecular weight larger than 200 kDa, 4% gel is better.

| 8% Phos-tag gel | Final concentration | Amount |
|-----------------|---------------------|--------|
| 1.5 M Tris-HCl pH 8.8 | 0.375 M | 1.25 mL |
| 30% Acryl/Bis | 8% | 1.335 mL |
| 10% SDS | 0.1% | 50 μL |
| 10 mM MnCl₂ | 150 μM | 75 μL |
| 5 mM Phos-tag Acrylamide | 75 μM | 75 μL |
| 10% APS | 0.05% | 25 μL |
| TEMED | N/A | 5 μL |
| ddH₂O | N/A | 2.185 mL |
| Total | N/A | 5 mL |

Freshly prepared.

| 10 × TGS buffer | Final concentration | Amount |
|-----------------|---------------------|--------|
| Trizma base | 250 mM | 30.3 g |
| Glycine | 1.92 M | 144 g |
| SDS | 1% | 10 g |
| ddH₂O | N/A | to 1 L |
| Total | N/A | 1 L |

Store at 15°C–25°C for 1 year.

| 1 × TGS buffer | Final concentration | Amount |
|-----------------|---------------------|--------|
| 10 × TGS buffer | 10% | 100 mL |
| ddH₂O | N/A | 900 mL |
| Total | N/A | 1 L |

Store at 15°C–25°C for 1 year, and pre-cool at 4°C before use.

| 10 × Transfer buffer (methanol-free) | Final concentration | Amount |
|-------------------------------------|---------------------|--------|
| Trizma base | 250 mM | 30.3 g |
| Glycine | 1.92 M | 144 g |
| ddH₂O | N/A | to 1 L |
| Total | N/A | 1 L |

Store at 15°C–25°C for 1 year.
### 1 × Transfer buffer (containing methanol)

| Reagent                        | Final concentration | Amount   |
|--------------------------------|---------------------|----------|
| 10 × Transfer buffer (methanol-free) | 10%                | 100 mL   |
| Methanol                       | 20%                 | 200 mL   |
| ddH₂O                           | N/A                 | to 1 L   |
| Total                           | N/A                 | 1 L      |

Store at 15°C–25°C, and pre-cool at 4°C before use.

### Phos-tag transfer buffer

| Reagent   | Final concentration | Amount   |
|-----------|---------------------|----------|
| TGS buffer| N/A                 | 39.2 mL  |
| 0.5 M EDTA pH 8.0 | 1 mM       | 800 µL   |
| Total     | N/A                 | 40 mL    |

Freshly prepared.

### 1 × PBS buffer

| Reagent      | Final concentration | Amount   |
|--------------|---------------------|----------|
| 10 × PBS buffer | 10%             | 100 mL   |
| ddH₂O         | N/A                 | 900 mL   |
| Total         | N/A                 | 1 L      |

Store at 15°C–25°C for 1 year, and pre-cool at 4°C before use.

### 10 × PBS buffer

| Reagent          | Final concentration | Amount   |
|------------------|---------------------|----------|
| NaCl             | 8%                  | 80 g     |
| KH₂PO₄           | 0.2%                | 2 g      |
| Na₂HPO₄·12H₂O    | 3.58%               | 35.8 g   |
| KCl              | 0.2%                | 2 g      |
| ddH₂O            | N/A                 | to 1 L   |
| Total            | N/A                 | 1 L      |

Store at 15°C–25°C for 1 year.

### 1 × PBST buffer

| Reagent        | Final concentration | Amount   |
|----------------|---------------------|----------|
| 10 × PBS Buffer| 1 ×                 | 100 mL   |
| Tween-20       | N/A                 | 3.125 mL |
| ddH₂O          | N/A                 | 900 mL   |
| Total          | N/A                 | 1 L      |

Store at 15°C–25°C for 1 year.

### 10% APS (Ammonium Persulfate)

| Reagent | Final concentration | Amount   |
|---------|---------------------|----------|
| APS     | 10%                 | 1 g      |
| ddH₂O   | N/A                 | to 10 mL |
| Total   | N/A                 | 50 mL    |

Store at −20°C for up to 1 month or store at 4°C for up to 1 week.
### 10% SDS (sodium dodecyl sulfate)

| Reagent          | Final concentration | Amount   |
|------------------|---------------------|----------|
| SDS              | 10%                 | 10 g     |
| ddH₂O            | N/A                 | to 100 mL|
| Total            | N/A                 | 100 mL   |

Store at 15°C–25°C for 1 year.

### 10 mM MnCl₂

| Reagent         | Final concentration | Amount   |
|-----------------|---------------------|----------|
| MnCl₂·4H₂O      | 10 mM               | 0.1 g    |
| ddH₂O           | N/A                 | 42 mL    |
| Total           | N/A                 | 50 mL    |

Store at 15°C–25°C for 1 year.

### 5% skim milk

| Reagent       | Final concentration | Amount   |
|---------------|---------------------|----------|
| Skim milk     | 5%                  | 1 g      |
| 1 x PBST      | N/A                 | to 20 mL |
| Total         | N/A                 | 20 mL    |

Freshly prepared.

### 2.5 M NaCl

| Reagent     | Final concentration | Amount   |
|-------------|---------------------|----------|
| NaCl        | 2.5 M               | 14.61 g  |
| ddH₂O       | N/A                 | to 100 mL|
| Total       | N/A                 | 100 mL   |

Autoclave at 121°C, 20 min and store at 15°C–25°C for 1 year.

### 0.5 M EDTA pH 8.0

| Reagent       | Final concentration | Amount   |
|---------------|---------------------|----------|
| Na₂EDTA·2H₂O  | 0.5 M               | 18.61 g  |
| NaOH          | N/A                 | to pH = 8.0|
| ddH₂O         | N/A                 | to 100 mL |
| Total         | N/A                 | 100 mL   |

Store at 15°C–25°C for 1 year.

### 1 M DTT

| Reagent | Final concentration | Amount   |
|---------|---------------------|----------|
| DTT     | 1 M                 | 3.09 g   |
| NaOAc   | N/A                 | 5 mL     |
| Total   | N/A                 | 5 mL     |

Filter with 0.22 μm filter and store at −20°C for 1 year.

### Lysozyme solution

| Reagent    | Final concentration | Amount   |
|------------|---------------------|----------|
| Lysozyme   | 5%                  | 5 mg     |
| ddH₂O      | N/A                 | to 100 μL|
| Total      | N/A                 | 100 μL   |

Freshly prepared.
### 100 mM PMSF

| Reagent         | Final concentration | Amount     |
|-----------------|---------------------|------------|
| PMSF            | 100 mM              | 0.174 g    |
| Ethanol absolute| N/A                 | to 10 mL   |
| **Total**       | N/A                 | 10 mL      |

Filter with 0.22 μm filter and store at −20°C for 1 year.

### 500 mM IPTG

| Reagent | Final concentration | Amount     |
|---------|---------------------|------------|
| IPTG    | 500 mM              | 1.192 g    |
| ddH₂O   | N/A                 | to 10 mL   |
| **Total** | N/A             | 10 mL      |

Filter with 0.22 μm filter and store at −20°C for 1 year.

### 1 M Tris-HCl pH 6.8

| Reagent         | Final concentration | Amount     |
|-----------------|---------------------|------------|
| Trizma base     | 1 M                 | 12.11 g    |
| HCl             | N/A                 | to pH = 6.8|
| ddH₂O           | N/A                 | to 100 mL  |
| **Total**       | N/A                 | 100 mL     |

Store at 15°C–25°C for 1 year.

### 1 M Tris-HCl pH 7.4

| Reagent         | Final concentration | Amount     |
|-----------------|---------------------|------------|
| Trizma base     | 1 M                 | 12.11 g    |
| HCl             | N/A                 | to pH = 7.4|
| ddH₂O           | N/A                 | to 100 mL  |
| **Total**       | N/A                 | 100 mL     |

Store at 15°C–25°C for 1 year.

### 1 M Tris-HCl pH 7.5

| Reagent         | Final concentration | Amount     |
|-----------------|---------------------|------------|
| Trizma base     | 1 M                 | 12.11 g    |
| HCl             | N/A                 | to pH = 7.5|
| ddH₂O           | N/A                 | to 100 mL  |
| **Total**       | N/A                 | 100 mL     |

Store at 15°C–25°C for 1 year.

### 1 M Tris-HCl pH 8.0

| Reagent         | Final concentration | Amount     |
|-----------------|---------------------|------------|
| Trizma base     | 1 M                 | 12.11 g    |
| HCl             | N/A                 | to pH = 8.0|
| ddH₂O           | N/A                 | to 100 mL  |
| **Total**       | N/A                 | 100 mL     |

Store at 15°C–25°C for 1 year.
### GST-lysis buffer

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| 1 M Tris-HCl pH 8.0      | 50 mM               | 5 mL    |
| 0.5 M EDTA pH 8.0        | 1 mM                | 0.2 mL  |
| 2.5 M NaCl               | 100 mM              | 4 mL    |
| ddH₂O                    | N/A                 | to 100 mL |
| **Total**                | **N/A**             | **100 mL** |

Store at 4°C for 1 year.

### MBP column buffer

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| 1 M Tris-HCl pH 7.4      | 20 mM               | 10 mL   |
| 2.5 M NaCl               | 200 mM              | 40 mL   |
| 0.5 M EDTA               | 1 mM                | 1 mL    |
| ddH₂O                    | N/A                 | to 500 mL |
| **Total**                | **N/A**             | **500 mL** |

Store at 4°C for 1 year.

### GST elution solution

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| 1 M Tris-HCl pH 8.0      | 5 mM                | 50 μL   |
| L-Glutathione reduced    | 10 mM               | 3 mg    |
| ddH₂O                    | N/A                 | to 1 mL |
| **Total**                | **N/A**             | **1 mL** |

Freshly prepared.

### MBP elution solution

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| D- (+)-Maltose monohydrate| 10 mM              | 3.6 mg  |
| MBP column buffer        | N/A                 | to 1 mL |
| **Total**                | **N/A**             | **1 mL** |

Freshly prepared.

### Coomassie brilliant blue G-250 staining solution

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Coomassie brilliant blue G-250 | 1%                | 1 g     |
| Methanol                 | 40%                 | 40 mL   |
| Acetic acid              | 10%                 | 10 mL   |
| ddH₂O                    | N/A                 | to 100 mL |
| **Total**                | **N/A**             | **100 mL** |

Store at 15°C–25°C for 1 year.

### Decolored solution

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| Ethanol absolute         | 5%                  | 25 mL   |
| Acetic acid              | 10%                 | 50 mL   |
| ddH₂O                    | N/A                 | 425 mL  |
| **Total**                | **N/A**             | **500 mL** |

Store at 15°C–25°C for 1 year.
**STEP-BY-STEP METHOD DETAILS**

**Protein expression and purifications**

© Timing: 3 days

Induce and purify GST-AFC2 kinase and MBP-RSZ21 substrate protein, respectively.

1. Induce GST-AFC2 protein expression.
   a. Streak a GST-AFC2 single colony into 5 mL of LB liquid medium supplemented with 50 μg/mL ampicillin and incubate at 37°C for 12–14 h at 230 rpm.
   b. Transfer all the bacterial culture into 300 mL of LB liquid medium supplemented with 50 μg/mL ampicillin and incubate at 37°C until OD$_{600}$ = 0.6–0.8.
   c. Add 600 μL of 500 mM IPTG into 300 mL bacterial culture for the final IPTG concentration is 1 mM and incubate them at 37°C for 3 h at 230 rpm to induce protein expression.
   d. Centrifuge at 4°C for 10 min at 926 g to collect pellets.

2. Induce MBP-RSZ21 protein expression.
a. Streak a MBP-RSZ21 single colony into 5 mL of LB liquid medium supplemented with 50 μg/mL ampicillin and incubate at 37°C for 12–14 h at 230 rpm.

b. Transfer all the bacterial culture into 300 mL of LB liquid medium supplemented with 50 μg/mL ampicillin and incubate at 37°C at 230 rpm until OD₆₀₀ = 0.6–0.8.

c. Add 600 μL of 500 mM IPTG in 300 mL bacterial culture for the IPTG final concentration is 1 mM and incubate them at 30°C for 3 h at 230 rpm to induce protein expression.

d. Centrifuge at 4°C for 10 min at 926 g to collect pellets.

[Pause point: At this step, bacterial pellets can be stored at -80°C for one week until processing protein purifications.]

3. Purify GST-AFC2 protein and MBP-RSZ21 protein.

   a. Add 20 mL of GST-lysis buffer into GST-AFC2 pellets and 20 mL of MBP column buffer into MBP-RSZ21 pellets, respectively. Transfer them into 50 mL centrifuge tubes and vortex to suspend bacteria pellets.

   Note: Pellets should be completely suspended.

   b. Add 200 μL of 100 mM PMSF, 200 μL of TritonX-100 and 50 μL of lysozyme solution into the suspended pellets, and then incubate at 4°C for 1 h with gentle shaking.

   Note: Proteinase inhibitor PMSF has short half-life (~ 30 min) in the aqueous solution. Add 200 μL of 100 mM PMSF every half hour throughout the purification process.

   c. Keep the 50 mL centrifuge tubes on ice and lyse bacteria cells under sonication.

   Note: In this protocol, we used SONICS Ultrasonic Cell crusher (total sonication in 18–25 cycles, for each cycle: sonicate 15 s and pause 45 s).

   d. Centrifuge at 4°C for 18 min at 14,811 g and then collect supernatant.

   e. Pre-clean columns. For GST-AFC2 protein purification, wash the column with 10 mL of 1× PBS buffer for 3 times and then add 30 μL of glutathione Sepharose (Cytiva, Cat# 17075601) to the column. For MBP-RSZ21 protein purification, wash the column with MBP column buffer for 3 times and then add 30 μL of amylose resin (New England Biolabs, Cat# E8021S) to the column, wash with 10 mL of MBP column buffer for 4–5 times (invert to mix and then flow through the column).

   Note: Because the glutathione sepharose beads are stored in 20% ethanol, flow through the supernatant first and then wash with 10 mL of 1× PBS buffer for 4–5 times (invert to mix and then flow through the column).

   Note: It is recommended to do the protein purification experiments (start from this step) in a cold room. Otherwise, keep all the solutions and columns cool (prepare solutions and store them in a 4°C freezer in advance).

   f. Transfer all the supernatant into the corresponding purification column.

   Note: The pellet in the 50 mL centrifuge tube contains bacterial debris. When transferring supernatant, use a disposable plastic pipette to gently pick up supernatant.

   g. Incubate the supernatant with beads at 4°C for 2 h with gentle shaking.

   Note: Prepare 10% separating gel and stacking gel at this time.
h. Flow through the supernatant out of column.

i. Wash columns. For GST-AFC2 purification, wash the column with 10 mL of 1 × PBS buffer for 4–5 times. For MBP-RS21 protein purification, wash the column with MBP column buffer for 4–5 times.

j. Prepare 1 mL of elution solution.

**Note:** The elution solution varies according to the fusion tags and must be freshly prepared before use.

k. For protein elution, gently place 200 μL of elution solution to the column bottom and maintain for 1–2 min, then flow through the solution into a 1.5 mL Eppendorf tube (collection Eppendorf tube), label this Eppendorf tube as elution 1 and place this tube on ice immediately. Repeat this procedure for five time, to obtain five individual protein elution samples (elution 1–5).

**Note:** Remove the washing buffer as much as possible. We used to keep the column vertical and sometimes close and open the column cap for more times to impose air pressure for pushing the washing buffer out of the column.

**Note:** Eppendorf tubes should be labeled in advance to save time.

l. Transfer 12 μL of eluted protein sample from each collection Eppendorf tube into another 1.5 mL Eppendorf tube, add 4 μL of 4 × SDS loading buffer and place the Eppendorf tube at 100°C for 10 min.

**Note:** Turn on the water bath or heater in advance.

m. Perform routine SDS-PAGE electrophoresis to check the protein purification result.

n. Coomassie brilliant blue stain for 1–2 h and then decolorizing for 8–12 h with decolored solution, image the gel and compare the protein purity and concentration among all the eluted samples.

**Note:** After protein purification, it is recommended to carry out the downstream kinase assay immediately. Otherwise, the eluted protein samples can be aliquoted and stored in −80°C. Try to minimize the freeze and thaw cycles.

**Note:** The concentration and purity of each eluted protein is not identical. In general, the concentration of the first elution is higher, but its purity is lower. One simple way to determine the protein concentration is to compare the corresponding band with the molecular weight marker. The separated band in the protein marker has its own concentration according to the product manual. For the purity issue, we choose the eluted sample with only one specific band on the gel. However, it is not uncommon to come across samples with multiple non-specific bands. They are still fine for downstream kinase assays when control experiments are properly included.

|| Pause point: The eluted proteins can be stored at −80°C.

**Kinase reaction**

© Timing: 2–3 h

Monitor the GST-AFC2 kinase activity under different temperatures or chemical inhibitor treatment. The phosphorylation levels of MBP-RS21 are characterized to determine the AFC2 kinase activities (Lin et al., 2022).
4. GST-AFC2 kinase assays at different temperatures.
   a. Experiment set up: test the kinase reactions at four different temperatures (22°C (positive control), 27°C, 32°C, 37°C).
   b. Add 8 μg of GST-AFC2 protein, 2 μg of MBP-RSZ21 protein and 80 μL of kinase reaction ATP buffer in a 1.5 mL Eppendorf tube for kinase reactions. We also set up a non-reaction negative control (Add 8 μg of GST-AFC2 protein, 2 μg of MBP-RSZ21 protein and 80 μL of kinase reaction ATP-free buffer in a 1.5 mL Eppendorf tube) at 22°C.

   Note: The exact protein concentration is determined with spectrophotometer.

c. Put reaction Eppendorf tubes into Eppendorf ThermoMixer at different temperatures for 1 h (without shaking).

d. Add 40 μL of 4 × SDS loading buffer and place the Eppendorf tubes at 100°C for 10 min to stop reactions.

   Note: Try to start reactions simultaneously.

   Pause point: The samples can be used for subsequent experiments immediately or stored at −80°C.

5. GST-AFC2 kinase assays with chemical treatment.
   a. Experiment set up: test the kinase reactions under different dosage (30 μM or 100 μM) of TG-003 or Dimethyl sulfoxide (DMSO, mock) treatment.
   b. Add 8 μg of GST-AFC2 protein and different dosage of TG-003 or DMSO and 80 μL of kinase reaction ATP buffer in 1.5 mL Eppendorf tube. We also set up a positive control (8 μg of GST-AFC2 protein and 80 μL of kinase reaction ATP buffer) without TG-003 or DMSO and a non-reaction negative control (8 μg of GST-AFC2 protein and 80 μL of kinase reaction ATP-free buffer).
   c. Keep Eppendorf tubes at 22°C for 30 min for pre-reactions.
   d. Add 8 μg of MBP-RSZ21 protein in pre-reaction Eppendorf tubes for kinase reactions.
   e. Keep Eppendorf tubes at 22°C for 1 h for kinase reactions.
   f. Add 40 μL of 4 × SDS loading buffer and place Eppendorf tubes at 100°C for 10 min to stop reactions.

   Note: TG-003 is a reported CLK kinase inhibitor (Muraki et al., 2004; Haltenhof et al., 2020).

   Pause point: The sample can be used for subsequent experiments immediately or stored at −80°C.

Activity detection

Timing: 2 days

Run kinase reaction products on regular SDS-PAGE or Phos-tag SDS-PAGE.

6. Perform SDS-PAGE electrophoresis as loading controls.
7. Perform Phos-tag SDS-PAGE electrophoresis to separate the phosphorylated bands and non-phosphorylated bands.
   a. Prepare 8% Phos-tag gel and regular stacking gel.
   b. Load samples into gel (from left lane to right lane): 5 μL of prestained protein marker, 5 μL of WIDE-VIEW prestained protein size marker III, 10 μL of each reaction product, 5 μL of WIDE-VIEW prestained protein size marker III and 5 μL of prestained protein marker.
   c. Perform the Phos-tag SDS-PAGE electrophoresis at 80 V in a cold room or on ice.
Note: In general, perform the electrophoresis until the bromophenol dye reaches gel bottom edge. Electrophoresis time can be adjusted according to the actual protein molecular weight. The protein samples move relatively slow in the Phos-tag gel than in the stacking gel. Therefore you would perform the Phos-tag SDS-PAGE electrophoresis at 80 V for about 6–8 h.

Note: Phos-tag SDS-PAGE is very sensitive to chelating agents such as EDTA. Avoid preparing samples with reagent containing EDTA, and use EDTA-free buffer when performing Phos-tag SDS-PAGE.

Note: Due to the feature of phos-tag reagent, the regular prestained protein marker cannot separate well, commercial WIDE-VIEW prestained protein size marker III is recommended. But WIDE-VIEW staineded protein size marker cannot estimate molecular weight, so regular prestained protein marker is also required. If there are empty lanes, try to load the same volume of 4 × SDS loading buffer.

d. After electrophoresis, cut the whole gel and wash the gel with 20 mL of Phos-tag transfer buffer with gentle shaking for 10 min. Repeat this washing step twice.

Note: Prepare a square plastic petri dish for washing the gel with Phos-tag transfer buffer. The EDTA contained in the Phos-tag transfer buffer can chelate Mn2+ and improve the efficiency of protein transfer.

Note: The concentration range of EDTA is 1–10 mM, and its concentration can be chosen according to the experiment.

e. Wash the gel with 20 mL of 1 × transfer buffer for 10 min with gentle shaking in a cold room (4°C).

Note: 1 × transfer buffer was used to clean the EDTA chelated with metal ions on the gel surface to improve the efficiency of protein transfer.

f. Transfer proteins to nitrocellulose membrane (NC membrane) using wet transferring system at 100 V for 60 min on ice.

Note: Keeping the protein transfer tank on ice prevents temperature rise and improve the protein transfer efficiency.

g. Block the membrane with 5% skim milk in a cold room (4°C) for 2 h or overnight (8–12 h) with gentle shaking.

h. Wash the membrane with 1 × PBST buffer briefly.

i. Incubate with the primary antibody against the target protein in 1 × PBST buffer in a cold room (4°C) for 2 h or overnight (8–12 h) with gentle shaking.

j. Wash the membrane with 15 mL of 1 × PBST buffer for 10 min in a cold room (4°C) with gentle shaking. Discard the buffer, and repeat washing for three times.

k. Incubate the membrane with corresponding secondary antibody in 1 × PBST buffer for 1 h in a cold room (4°C).

l. Wash the membrane with 15 mL of 1 × PBST buffer for 10 min in a cold room (4°C) with gentle shaking. Discard the buffer, and repeat washing for three times.

m. Add 400 μL of mixed enhanced chemiluminescence (ECL) solution to the membrane.

Note: The ECL solution consists of solution A and solution B. 200 μL of solution A and 200 μL of solution B were mixed in a petri dish. Use a disposable pipette to gently cover the mixed ECL solution on the membrane surface for about 10 times.
If fluorescent secondary antibody is used, the ECL solution is not required.

n. Remove excess ECL solution.

Note: The dilution ratio of the primary antibody and the secondary antibody should be optimized according to the antibody sensitivity and protein concentrations.

Note: The ECL solution treatment time varies among different suppliers. Refer to the product manual and adjust the reaction time accordingly.

Note: The buffer and the Phos-tag gel used for the Phos-tag SDS-PAGE electrophoresis must be freshly prepared.

o. The luminescent signal on the membrane was recorded with a charge-coupled device (CCD) based gel imaging system.

Alternatives: The luminescent signal on the membrane is also suitable for a traditional film development system. If film developer machine and dark room are available, you can use this system to detect signals on the membrane.

EXPECTED OUTCOMES
First, we monitored AFC2 kinase activities under different temperatures (Figure 1). The SDS-PAGE gels served as loading controls. From the Phos-tag gel, we could distinguish the phosphorated forms and the non-phosphorated forms of RSZ21 proteins. In the absence of ATP, the phosphorated forms of RSZ21 were absent, indicating that at this circumstance, AFC2 could not phosphorylate its substrate RSZ21. However, when the reaction temperatures increased, the phosphorated forms of RSZ21 were reduced. Therefore, we concluded that high temperature repressed AFC2 kinase activities.

Second, we compared the effect of small molecule treatment on AFC2 kinase activities. In different dosages of TG-003 treatment but not DMSO solvent treatment, the bands of phosphorated form of RSZ21 were gradually weaken (Figure 2). This result indicated that AFC2 kinase activities were negatively correlated with the TG-003 inhibitor concentrations.
LIMITATIONS

The approaches we described here are only suitable for monitoring in vitro kinase activities. For detecting in vivo kinase activities, it is recommended to refer to other assays (Prudent et al., 2010). Another limitation is that due to the tedious steps in western blot, it is unlikely to perform too many assays simultaneously. Therefore, for drug research and development, this assay is preferred to dissect the biochemical function of the potential chemicals, instead of using for high throughput screening.

TROUBLESHOOTING

**Problem 1**
Difficulty in protein induction (steps 1 and 2).

**Potential solution**
Only change a single parameter (IPTG concentration, temperature, E. coli strain, etc.) at every trial to explore the best induction conditions.

**Problem 2**
The OD600 of bacterial culture do not reach 0.6–0.8 in the shaking process (steps 1 and 2).

**Potential solution**
During bacterial culture, make sure all the instruments and reagents have been sterilized to avoid phage infection. It is also recommended to use a large volume flask (400 mL or 500 mL flask) for bacterial culture.

**Problem 3**
The target protein is not successfully purified (step 3).

**Potential solution**
If protein-of-interest can be induced but not purified, make sure the beads are successfully loaded on the column. Make sure the elution buffer is freshly made and PMSF is added frequently to avoid protein degradation. Another possibility is that the proteins form inclusion body in the pellet.
this circumstance, it is recommended to decrease the induction temperature and IPTG concentration for a mild protein induction environment.

Problem 4
Phosphorylated proteins are not detected (step 7).

Potential solution
Optimize the reaction system (the relative concentrations between kinase and substrate proteins and/or increase ATP concentrations) and reaction time (extend more time if necessary).

Problem 5
The phosphorylated bands and non-phosphorylated bands are too close (step 7).

Potential solution
Increase the concentration of Phos-tag acrylamide.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ziqiang Zhu (zqzhu@njnu.edu.cn).

Materials availability
All the non-commercial materials described in this study are available upon request.

Data and code availability
This study did not generate/analyze datasets.

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AUTHOR CONTRIBUTIONS
J.S. performed experiments and drafted the protocol. M.F. and R.W. participated in troubleshooting and discussions. Z.Z. designed research and revised the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Attwood, M.M., Fabbro, D., Sokolov, A.V., Knapp, S., and Schiöth, H.B. (2021). Trends in kinase drug discovery: targets, indications and inhibitor design. Nat. Rev. Drug Discov. 20, 839–861.

Ding, Y., Li, H., Zhang, X., Xie, Q., Gong, Z., and Yang, S. (2015). OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in. Dev. Cell 32, 278–289.

Haltenhof, T., Kotte, A., De Bortoli, F., Schiefer, S., Meineke, S., Emmerichs, A.K., Petermann, K.K., Timmermann, B., Imhof, P., Franz, A., et al. (2020). A conserved kinase-based body-temperature sensor globally controls alternative splicing and gene expression. Mol. Cell 78, 57–69.e4.

Lin, J., Shi, J., Zhang, Z., Zhong, B., and Zhu, Z. (2022). Plant AFC2 kinase desensitizes thermomorphogenesis through modulation of alternative splicing. iScience 25, 104051.

Mifflin, L., Ofengeim, D., and Yuan, J. (2020). Receptor-interacting protein kinase 1 (RIPK1) as a therapeutic target. Nat. Rev. Drug Discov. 19, 553–571.

Muraki, M., Ohkawara, B., Hosoya, T., Onogi, H., Koizumi, J., Koizumi, T., Sumi, K., Yomoda, J.I., Murray, M.V., Kimura, H., et al. (2004). Manipulation of alternative splicing by a newly developed inhibitor of Clks. J. Biol. Chem. 279, 24246–24254.

Overington, J.P., Al-Lazikani, B., and Hopkins, A.L. (2006). How many drug targets are there? Nat. Rev. Drug Discov 5, 993–996.

Prudent, R., Sautel, C.F., Moucadel, V., Laudet, B., Filhol, O., and Cochet, C. (2010). In vitro and in vivo assays of protein kinase CK2 activity. Methods Enzymol. 485, 597–610.