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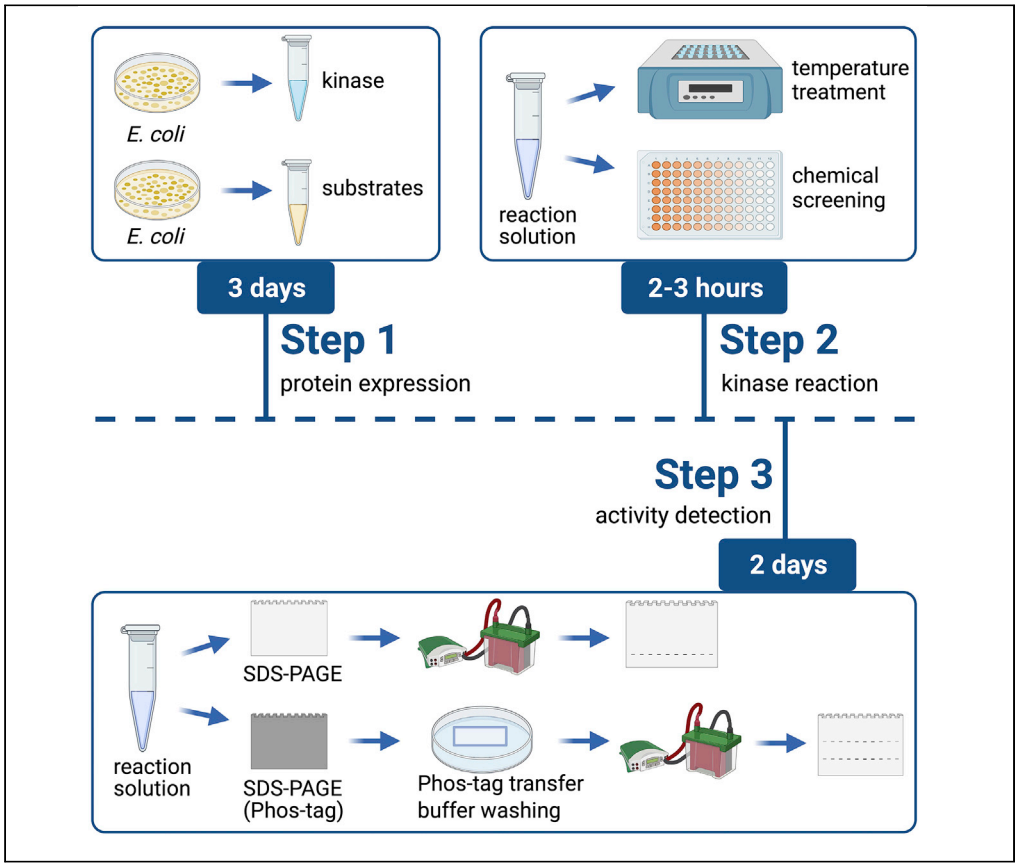
Protocol

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



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.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Producing kinase and substrate proteins from *E. coli* cells

Performing kinase reactions under treatments

Monitoring kinase activity with a Phos-tag technique

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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.

For complete details on the use and execution of this protocol, please refer to Lin et al. (2022).

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# C504-02
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 ($MnCl_2$)	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*)

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 ($MgCl_2$)	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# B546017-0500
N, N, N′, N′-Tetramethyl ethylenediamine (TEMED)	Sigma-Aldrich	Cat# V900853
Lysozyme	Solarbio	Cat# L8120
Tween-20	Sigma-Aldrich	Cat# V900548
TG-003	Sigma-Aldrich	Cat# T5575
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat# V900090
KCl	Sinopharm	Cat# 10016318
KH_2PO_4	Sinopharm	Cat# 10017618
$Na_2HPO_4 \cdot 12H_2O$	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-RSZ21	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

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
ddH_2O	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*)

Continued		
Reagent	Final concentration	Amount
HCl	N/A	to pH = 6.8
ddH_2O	N/A	to 100 mL
Total	**N/A**	**100 mL**
Store at 4°C for 1 year.		

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
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	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 ddH_2O 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.

8% Phos-tag gel		
Reagent	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 $MnCl_2$	150 μM	75 μL
5 mM Phos-tag Acrylamide	75 μM	75 μL
10% APS	0.05%	25 μL
TEMED	N/A	5 μL
ddH_2O	N/A	2.185 mL
Total	**N/A**	**5 mL**
Freshly prepared.		

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 $MnCl_2$ 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.

10 × TGS buffer		
Reagent	Final concentration	Amount
Trizma base	250 mM	30.3 g
Glycine	1.92 M	144 g
SDS	1%	10 g
ddH_2O	N/A	to 1 L
Total	**N/A**	1 L
Store at 15°C–25°C for 1 year.		

1 × TGS buffer		
Reagent	Final concentration	Amount
10 × TGS buffer	10%	100 mL
ddH_2O	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)		
Reagent	Final concentration	Amount
Trizma base	250 mM	30.3 g
Glycine	1.92 M	144 g
ddH_2O	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
ddH_2O	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
1 × 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
ddH_2O	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
KH_2PO_4	0.2%	2 g
$Na_2HPO_4 \cdot 12H_2O$	3.58%	35.8 g
KCl	0.2%	2 g
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	N/A	to 100 mL
Total	**N/A**	**100 mL**
Store at 15°C–25°C for 1 year.		

10 mM $MnCl_2$		
Reagent	Final concentration	Amount
$MnCl_2 \cdot 4H_2O$	10 mM	0.1 g
ddH_2O	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 × 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
ddH_2O	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
$Na_2EDTA \cdot 2H_2O$	0.5 M	18.61 g
NaOH	N/A	to pH = 8.0
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	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
ddH_2O	N/A	425 mL
Total	**N/A**	**500 mL**
Store at 15°C–25°C for 1 year.		

200 mM ATP		
Reagent	Final concentration	Amount
ATP	200 mM	0.121 g
1 M Tris-HCl pH 6.8	N/A	to 1 mL
Total	**N/A**	**1 mL**
Store at −20°C for 1 month.		

10 mM TG-003		
Reagent	Final concentration	Amount
TG-003	10 mM	5 mg
DMSO	N/A	2 mL
Total	**N/A**	**2 mL**
Store at −20°C for 1 year.		

Kinase reaction ATP buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl pH 7.5	25 mM	25 µL
200 mM $MgCl_2$	12 mM	6 µL
1 M DTT	1 mM	1 µL
200 mM ATP	1 mM	5 µL
ddH_2O	N/A	963 µL
Total	**N/A**	**1 mL**
Freshly prepared.		

Kinase reaction ATP-free buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl pH 7.5	25 mM	25 µL
200 mM $MgCl_2$	12 mM	6 µL
1 M DTT	1 mM	1 µL
ddH_2O	N/A	968 µL
Total	**N/A**	**1 mL**
Freshly prepared.		

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 at 230 rpm 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 OD_{600} = 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-RSZ21 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-RSZ21 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 stainededed 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 Mn^{2+} 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.

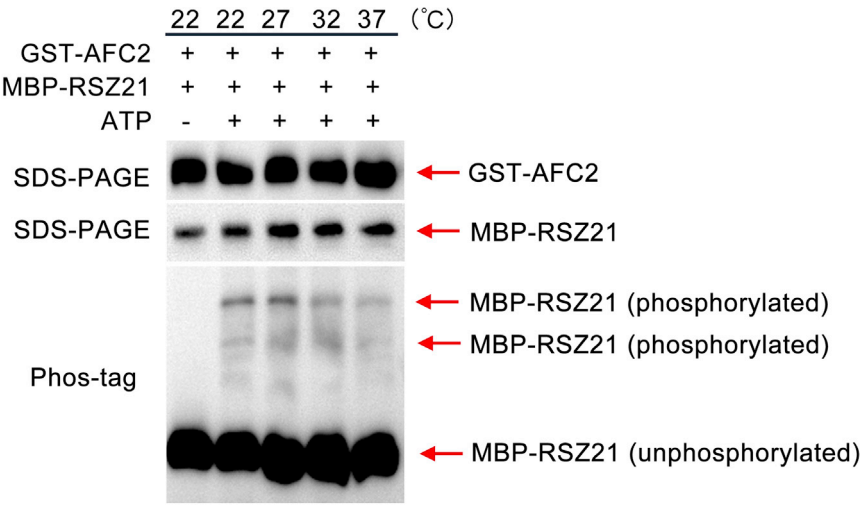

Figure 1. High temperature reduces AFC2 kinase activity

Note: 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.

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.

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

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.

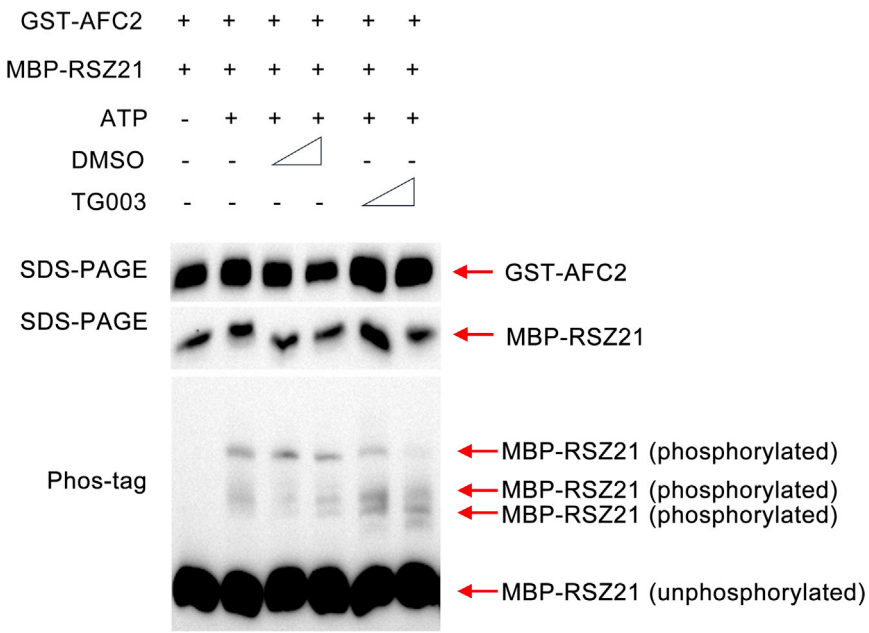

Figure 2. CLKs inhibitor TG-003 reduces AFC2 kinase activity

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 OD_{600} 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. Under

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.

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