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Characterization of ATG8-family protein phosphorylation by Phos-tag gel for autophagy study

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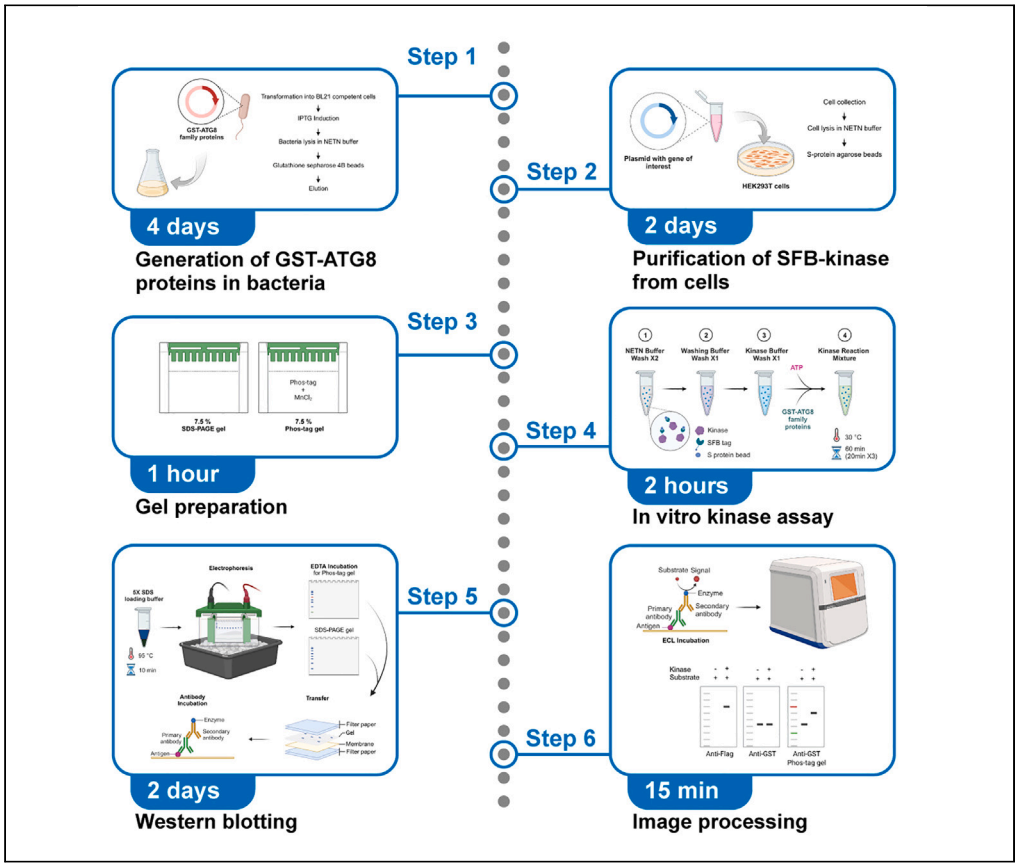

Autophagy supports cell survival under different stress conditions, where ATG8-family proteins are required for autophagosome biogenesis/maturation and selective autophagy. Here, we present a protocol for studying ATG8-family protein phosphorylation using phos-tag gel, a modified SDS-PAGE system, when the related phosphorylation site information and/or specific phospho-antibody are unavailable. We describe steps for generating GST-ATG8 proteins in bacteria, purifying SFB kinase from cells, preparing gel, and an *in vitro* kinase assay. We then detail procedures for western blotting and image processing.

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

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Highlights

In vitro purification of human ATG8-family autophagy proteins from bacteria

Purification of SFB-tagged kinases from cultured cells

Detection of ATG8-family protein phosphorylation via the phos-tag-based SDS-PAGE

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Characterization of ATG8-family protein phosphorylation by Phos-tag gel for autophagy study

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SUMMARY

Autophagy supports cell survival under different stress conditions, where ATG8-family proteins are required for autophagosome biogenesis/maturation and selective autophagy. Here, we present a protocol for studying ATG8-family protein phosphorylation using Phos-tag gel, a modified SDS-PAGE system, when the related phosphorylation site information and/or specific phospho-antibody are unavailable. We describe steps for generating GST-ATG8 proteins in bacteria, purifying S protein-Flag-SBP protein (SFB)-tagged kinasefrom cells, preparing gel, and an *in vitro* kinase assay. We then detail procedures for western blotting and image processing.

For complete details on the use and execution of this protocol, please refer to Seo et al.[1]

BEFORE YOU BEGIN

Before you begin, constructs encoding the recombinant kinase of interest and ATG8-family proteins need to be prepared. Here, we will take the S protein-Flag-SBP protein (SFB)-tagged kinase of interest (e.g., MAP4K2) and GST-tagged ATG8-family proteins (e.g., LC3A, LC3B) as an example to illustrate how Phos-tag gel can be used to analyze phosphorylation events for ATG8-family proteins. The protocol below describes specific steps for studying the phosphorylation of LC3A and LC3B via *in vitro* kinase assay. However, we have also used this protocol to analyze other ATG8-family proteins.

GST-ATG8-family protein preparation

Timing: 4 days

1. Prepare the constructs encoding glutathione-S-transferase (GST)-tagged LC3A and LC3B proteins. Here, we use the Gateway compatible GST-DEST vector to generate GST-LC3A and GST-LC3B plasmids using DH5α bacterial cells. Other GST-tagged vectors like pGEX plasmids can be also used.
2. Transform the GST-LC3A and GST-LC3B plasmids into competent BL21 bacterial cells.
3. Next day, pick and shake bacterial clones in 5 mL ampicillin-containing LB broth at 37°C for 12–16 h.

Note: Please check the information of antibiotic resistance for your constructs. We grow bacterial clones in ampicillin-containing LB broth because our GST-DEST vector is ampicillin resistant.

Optional: To accelerate the progress, bacterial clones can be shaken in 1–2 mL ampicillin LB broth at 37°C for 4–6 h and then move on to the protein induction steps as described below.

4. Transfer 200 μL bacteria into 5 mL ampicillin-containing LB broth and shake at 37°C until the O.D. (optical density at 600 nm) value reaches between 0.4–0.6.
5. Add 5 μL IPTG (0.5 M) for protein induction and shake bacteria at 30°C for 12–16 h.
6. Spin down the bacteria at 2,500 g for 15 min and discard the medium.
7. Add 0.5 mL NETN buffer with the protease inhibitors.
8. Suspend the bacterial pellet and transfer it into a 1.5 mL EP tube.
9. Sonicate the bacteria in cold room for 3 times with 15 s/time on 40% amplitude.

Note: Always keep the tubes on ice to avoid protein denaturation by heat.

10. Spin at 16,000 g at 4°C for 10 min and transfer the supernatant into a new 1.5 mL EP tube.

Note: Always keep the tubes on ice.

11. Add 25 μL glutathione Sepharose 4B beads and rotate the tubes in the cold room for at least 6 h.
12. Spin down the beads at 845 g for 30 s. Wash the beads with 1 mL NETN buffer with the protease inhibitors for 3 times.
13. Add 200–500 μL glutathione elution buffer with the protease inhibitors and rotate the tubes in cold room for 12–16 h.
14. Spin the tubes at 845 g at 4°C for 5 min and transfer the supernatant into a new 1.5 mL EP tube.
15. The purified GST-LC3A and GST-LC3B proteins can be examined by adding 2× SDS loading buffer for SDS-PAGE and Coomassie blue staining (Figure 1).
16. Measure the protein concentration using the standard methods like Bradford assay.

Note: If the amount of the purified protein is not enough, growing bacteria in a large scale (e.g., 100 mL) for protein purification is suggested.

SFB-kinase preparation

Timing: 2 days

17. Prepare the construct encoding SFB-tagged MAP4K2 kinase.
18. Seed HEK293T cells in 100 mm cell culture dishes and grow them in a standard incubator (i.e., 37°C, 5% CO2) for plasmid transfection.

Note: Low-passage cells usually yield good transfection efficiency and protein expression. Plasmid transfection should be performed within 24 h since cells are seeded in dishes.

19. Once cell density reaches 60%–70%, prepare Opti-MEM, and PEI solution (1 mg/mL).
20. Make a transfection mixture.
    Prepare two 1.5 mL tubes for each reaction.
    a. For tube 1, mix 500 μL Opti-MEM with 2∼5 μg SFB-MAP4K2 plasmid.
    b. For tube 2, mix 500 μL Opti-MEM with 30∼50 μL PEI.
    c. Combine tube 1 and tube 2 and incubate the mixture for 20 min.
    d. Add the mixture into HEK293T cells.
21. After 24 h, gently rinse the cells with 5 mL of ice-cold 1× PBS.
22. Add 1 mL ice-cold NETN buffer with the protease/phosphatase inhibitors into the dishes.
23. Collect cells using scraper and transfer all the cells into a 1.5 mL EP tube.

Note: Always keep the tube on ice.

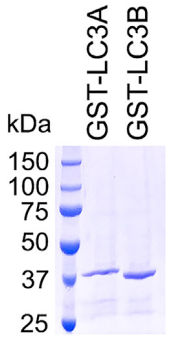

Figure 1. Preparation of GST-LC3A and GST-LC3B proteins for *in vitro* kinase assay

Bacterially purified GST-LC3A and GST-LC3B proteins are prepared for MAP4K2 *in vitro* kinase assay. The purified proteins are visualized by Coomassie blue staining.

24. Put the tube on the platform shaker in cold room for 20–30 min and spin down the insoluble pellet at 16,000 g at 4°C for 10 min.
25. Transfer the supernatant into a new 1.5 mL tube.
26. Add 25 μL of S-protein agarose beads into the tubes.

Note: Always keep the tubes on ice.

27. Rotate the tubes at 4°C for at least 3 h.

Preparation of SDS-PAGE gel

Timing: 60 min

28. Follow the table below to make the regular 7.5% resolving gel and Phos-tag-containing 7.5% resolving gel in 50 mL tubes.

Note: Before you add the resolving gel solution into the space between the two glass plates, use distilled water first to check the potential leakage issue.

⚠ CRITICAL: Add 10% APS and TEMED into the resolving and stacking gel solutions lastly. Once APS and TEMED are added, mix the solution well and transfer it into the space between the two glass plates immediately.

29. Mix the resolving gel solution well and transfer it into the space between the two glass plates.
30. Seal the gel with 1–2 mL Isopropanol to flatten the surface and remove bubbles.
31. Wait 30 min for gel polymerization.
32. Remove the Isopropanol.
33. Follow the table below to make the stacking gel solution in a 15 mL tube.
34. Mix the stacking gel solution well and transfer 1–2 mL stacking gel solution onto the top of the polymerized resolving gel.

⚠ CRITICAL: Add 10% APS and TEMED into the resolving and stacking gel solutions lastly. Once APS and TEMED are added, mix the solution well and transfer it into the space between the two glass plates immediately.

35. Insert 10 or 15-well combs.
36. Wait 30 min for gel polymerization.

Note: SDS-PAGE gels can be stored at 4°C for up to 2–3 weeks.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-GST (1:5,000 dilution)	Seo et al.[1]	N/A
Anti-Flag (1:3,000 dilution)	Sigma-Aldrich	Cat# F7425, RRID: AB_439687
Bacterial and virus strains		
DH5α and BL21 competent cells	Seo et al.[1]	N/A
Chemicals, peptides, and recombinant proteins		
Tris base	Fisher BioReagents	Cat# BP152-5
NaCl	Fisher BioReagents	Cat# S271-3
EDTA	Sigma-Aldrich	Cat# E6758-500G
NP-40	EMD Millipore	Cat# 492016-500ML
β-Glycerolphosphate	Sigma-Aldrich	Cat# G9422-100G
NaF	Sigma-Aldrich	Cat# S6776-100G
PMSF	Sigma-Aldrich	Cat# P7626
Sodium orthovanadate	Sigma-Aldrich	Cat# S6508-50G
Pepstatin A	Sigma-Aldrich	Cat# P-4265
Aprotinin	Sigma-Aldrich	Cat# A1153
Isopropyl β-d-1-thiogalactopyranoside (IPTG)	Fisher BioReagents	Cat# BP1620-10
Protein kinases buffer	Cell Signaling Technology	Cat# 9802
HEPES	Sigma-Aldrich	Cat# H3375-250G
Potassium acetate	Fisher Chemical	Cat# P171-500
$MgCl_2$	Fisher Chemical	Cat# M33-500
$MnCl_2$	Sigma-Aldrich	Cat# M3634-500G
SDS	Sigma-Aldrich	Cat# L5750-5KG
Glycerol	Sigma-Aldrich	Cat# G2025-1L
Bromophenol blue	Bio-Rad	Cat# 161-0404
β-Mercaptoethanol	Sigma-Aldrich	Cat# M6250-250ML
Methanol	Fisher BioReagents	Cat# BP1105-4
Non-fat milk	Apex	Cat# 20-241
Tween 20	Fisher BioReagents	Cat# BP337-500
Protogel	National Diagnostics	Cat# EC-890
4× Protogel resolving buffer	National Diagnostics	Cat# EC-892
Ammonium persulfate (APS)	Sigma-Aldrich	Cat# A3678
TEMED	Fisher BioReagents	Cat# BP150-100
Phos-tag acrylamide	FUJIFILM Wako Chemicals	Cat# AAL-107
Stacking buffer	National Diagnostics	Cat# EC-893
ATP	Abcam	Cat# ab146525
Whatman 3MM CHR filter paper	Cytiva	Cat# 3030-917
Immobilon-P transfer membrane	Millipore	Cat# IPVH00010
Bovine serum albumin	Sigma-Aldrich	Cat# A7030-500G
Glutathione Sepharose 4B	GE Healthcare	Cat# 17-0756-01
L-glutathione	Sigma-Aldrich	Cat# G4251-25G
Acetic acid	Fisher Chemical	Cat# A38C-212
Brilliant blue R-250	Fisher BioReagents	Cat# BP101-25
S-protein beads	Novagen	Cat# 69704
SuperSignal West Pico PLUS chemiluminescent substrate (ECL)	Thermo Scientific	Cat# 34578
Opti-MEM (1×)	Gibco	Cat# 31985-070
Polyethylenimine (PEI)	Polysciences	Cat# 23966
Precision Plus Protein all blue prestained protein standards	Bio-Rad	Cat# 1610373
Tri-color prestained protein marker (10–180 kDa)	Bioland Scientific LLC	Cat# PM01-01

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
GST-LC3A protein	This paper	N/A
GST-LC3B protein	This paper	N/A
Experimental models: Cell lines		
HEK293T	ATCC	Cat# CRL-3216
Recombinant DNA		
SFB-MAP4K2	Seo et al.[1]	N/A
GST-LC3A	Seo et al.[1]	N/A
GST-LC3B	Seo et al.[1]	N/A
Software and algorithms		
Image Lab	Bio-Rad	http://bio-rad.com
BioRender	BioRender	http://biorender.com
Other		
ChemiDoc MP imaging system	Bio-Rad	N/A
Semi-Dry Blotting System	Expedeon	Cat# EBU-4000

MATERIALS AND EQUIPMENT

NETN buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl (pH 8.0)	20 mM	80 mL
5 M NaCl	100 mM	80 mL
0.5 M EDTA	1 mM	8 mL
NP-40	0.5%	20 mL
ddH_2O	N/A	Make up to 4 L
Total	**N/A**	**4 L**
Storage condition: 4°C for up to 6 months.		

NETN buffer with the protease inhibitors		
Reagent	Final concentration	Amount
0.2 M PMSF	1 mM	1.2 mL
1 M Pepstatin A	1 mM	240 μL
1 M Aprotinin	1 mM	240 μL
NETN buffer	N/A	Make up to 240 mL
Total	**N/A**	**240 mL**
Store at 4°C for up to one week. Storage conditions for each stock solution: – 20°C for PMSF, Pepstatin A, and Aprotinin.		

NETN buffer with the protease/phosphatase inhibitors		
Reagent	Final concentration	Amount
1 M β-glycerophosphate	7.5 mM	1.8 mL
2 M NaF	8.3 mM	1 mL
0.2 M PMSF	1 mM	1.2 mL
1 M Sodium vanadate	1 mM	240 μL
1 M Pepstatin A	1 mM	240 μL
1 M Aprotinin	1 mM	240 μL
NETN buffer	N/A	Make up to 240 mL
Total	**N/A**	**240 mL**
Store at 4°C for up to one week. Storage conditions for each stock solution: 4°C for β-glycerophosphate and NaF; – 20°C for PMSF, Sodium Vanadate, Pepstatin A, and Aprotinin.		

Glutathione elution buffer		
Reagent	Final concentration	Amount
1.5 M Tris-HCl pH 8.8	150 mM	3 mL
L-Glutathione reduced	20 mM	185 mg
ddH_2O	N/A	Make up to 30 mL
Total	**N/A**	**30 mL**
After addition of glutathione, adjust pH to 8.0. Prepare the mixture before use.		

Coomassie blue staining solution		
Reagent	Final concentration	Amount
Methanol	50%	1 L
Acetic acid	10%	200 mL
Brilliant blue R-250	1 mg/mL	2 g
ddH_2O	N/A	Make up to 2 L
Total	**N/A**	**2 L**
Store indefinitely at 20–25°C.		

Destaining solution		
Reagent	Final concentration	Amount
Methanol	15%	300 mL
Acetic acid	15%	300 mL
ddH_2O	N/A	1.4 L
Total	**N/A**	**2 L**
Store indefinitely at 20–25°C.		

Wash buffer		
Reagent	Final concentration	Amount
1 M HEPES (pH 7.4)	40 mM	4 mL
5 M NaCl	200 mM	4 mL
ddH_2O	N/A	92 mL
Total	**N/A**	**100 mL**
Store at 4°C for up to one month.		

Kinase assay buffer		
Reagent	Final concentration	Amount
1 M HEPES (pH 7.4)	30 mM	3 mL
1 M potassium acetate	50 mM	5 mL
1 M $MgCl_2$	5 mM	0.5 mL
ddH_2O	N/A	91.5 mL
Total	**N/A**	**100 mL**
Store at 4°C for up to one month. **Note:** Protein kinases buffer is also commercially available and can be used.		

2× SDS sample buffer		
Reagent	Final concentration	Amount
1 M Tris (pH 6.8)	100 mM	5 mL
20% SDS	4%	10 mL
Glycerol	20%	10 mL

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
Bromophenol blue	2 mg/mL	100 mg
β -mercaptoethanol	10%	5 mL
ddH_2O	N/A	Make up to 50 mL
Total	**N/A**	**50 mL**

Store indefinitely at 20–25°C.

⚠ CRITICAL: Precaution for safe handling of β-mercaptoethanol: Avoid contact with skin and eyes. Avoid inhalation of vapor or mist.

5× SDS sample buffer

Reagent	Final concentration	Amount
1 M Tris (pH 6.8)	250 mM	12.5 mL
SDS	100 mg/mL	5 g
Glycerol	50%	25 mL
Bromophenol blue	2.5 mg/mL	125 mg
β-mercaptoethanol	25%	12.5 mL
ddH_2O	N/A	Make up to 50 mL
Total	**N/A**	**50 mL**

Store indefinitely at 20–25°C.

⚠ CRITICAL: Precaution for safe handling of β-mercaptoethanol: Avoid contact with skin and eyes. Avoid inhalation of vapor or mist.

7.5% resolving gel mixture for 2 gels

Reagent	Final concentration	Amount
Protogel	7.5%	3 mL
Resolving gel buffer	N/A	2.4 mL
ddH_2O	N/A	6.6 mL
10% APS	0.6%	75 μL
TEMED	0.08%	10 μL
Total	**N/A**	**12.1 mL**

Store at 4°C for up to 2 weeks.

Note: Add 10% APS and TEMED at last. Once APS and TEMED were added, mix the resolving gel solution well and add it between the two glass plates immediately.

Phos-tag-containing 7.5% resolving gel mixture for 2 gels

Reagent	Final concentration	Amount
Protogel	7.5%	3 mL
Resolving gel buffer	N/A	2.4 mL
ddH_2O	53.7%	6.6 mL
10% APS	0.6%	75 μL
TEMED	0.08%	10 μL
5 mM Phos-tag	24.4 μM	60 μL
10 mM $MnCl_2$	97.6 μM	120 μL
Total	**N/A**	**12.3 mL**

Prepare the mixture before use.

Note: Add 10% APS and TEMED at last. Once APS and TEMED are added, mix the resolving gel solution well and add it into the space between the two glass plates immediately.

Stacking gel mixture for 2 gels		
Reagent	Final concentration	Amount
Protogel	4.8%	0.66 mL
Stacking Buffer	N/A	0.8 mL
ddH_2O	N/A	2.5 mL
10% APS	0.7%	25 μL
TEMED	0.1%	3.75 μL
Total	**N/A**	**4 mL**
Prepare the mixture before use.		

Note: Add 10% APS and TEMED at last. Once APS and TEMED are added, mix the stacking gel solution well and add it onto the top of the polymerized resolving gel immediately.

10× TGS buffer		
Reagent	Final concentration	Amount
Tris base	24.8 mM	6 g
Glycine	191.8 mM	28.8 g
Methanol	10%	200 mL
ddH_2O	N/A	Make up to 2 L
Total	**N/A**	**2 L**
Store at 20–25°C for up to one year.		

Transfer buffer		
Reagent	Final concentration	Amount
Glycine	191.8 mM	57.6 g
Tris base	253.6 mM	122.9 g
SDS	1.3 mM	1.5 g
Methanol	15%	600 mL
ddH_2O	N/A	Make up to 4 L
Total	**N/A**	**4 L**
Store at 20–25°C for up to one year.		

20× TBST buffer (pH 8.0)		
Reagent	Final concentration	Amount
Tris base	1 M	121.14 g
NaCl	3 mM	175 g
Tween 20	4%	40 mL
ddH_2O	N/A	Make up to 1 L
Total	**N/A**	**1 L**
Store at 20–25°C for up to one year.		

5% non-fat milk		
Reagent	Final concentration	Amount
Non-fat milk	5%	50 g
20× TBST	5%	50 mL
ddH_2O	N/A	Make up to 1 L
Total	**N/A**	**1 L**
Store at 4°C for up to one week.		

STEP-BY-STEP METHOD DETAILS

In vitro kinase assay

Timing: 2 h

In this section, the purified GST-LC3A/LC3B and SFB-MAP4K2 will be used for *in vitro* kinase assay.

1. Take the tubes containing the cell lysates with SFB-MAP4K2 and S-protein beads from the rotator and put them on ice.

   Note: Always keep the tubes on ice.

2. Wash the beads twice with ice-cold NETN buffer with the protease/phosphatase inhibitors, once with the ice-cold wash buffer, and once with ice-cold kinase assay buffer.
   For each wash,
   a. spin down the beads at 845 g for 30 s.
   b. remove the buffer using a vacuum or 1 mL-scale pipette.
   c. add 1 mL of proper buffer as mentioned above to repeat the wash.

   Note: After the last wash, the remaining kinase buffer can be further removed using a 20 μL-scale pipette.

3. Prepare the kinase reaction mixture following the table below and keep it on ice.

Kinase reaction mix	
Reagent	Amount (for one reaction)
GST-LC3A or GST-LC3B (0.1 μg/μL)	2 μL
500 μM ATP (10 mM)	1.5 μL
Kinase assay buffer	26.5 μL
Total	30 μL

   Note: The ratio of kinase and substrate is important. To improve the mobility shift of the phosphorylated GST-LC3A or GST-LC3B protein in Phos-tag gel, sufficient kinase should be prepared and used for the kinase assay to allow most of the GST-LC3A or GST-LC3B protein to be phosphorylated.

4. Add 30 μL of kinase reaction mixture into one S-protein beads-containing tube.
5. Incubate the tube in 30°C water bath for 1 h.

   Note: Gently tap the tubes every 20 minutes to resuspend the beads to improve the reaction.

6. Add 10 μL 5× SDS sample buffer into each tube and treat the tube samples on a heat block (95°C) for 10 min.
7. Spin the tubes at 16,000 g for 1 min and transfer the supernatant to a new 1.5 mL tube.

   Pause point: Samples can be stored at 4°C for 2–3 days. But for long-term storage, we recommend freezing samples at −20°C.

Western blotting

Timing: 2 days

In this section, the prepared protein samples will be transferred to PVDF membrane after SDS-PAGE, followed by incubating with the indicated primary and secondary antibodies.

8. Prepare two regular 7.5% gels for checking protein expression (i.e., SFB-MAP4K2 and GST-LC3A/LC3B) and one Phos-tag-containing 7.5% gel for checking protein phosphorylation (i.e., GST-LC3A/LC3B).
9. Load protein marker into the first lane of each gel.

Note: For Phos-tag gel, we usually use the tri-color protein marker (Bioland Scientific LLC) for better protein size indication.

10. Load 8∼10 μL protein sample into each well and fill the blank wells with 1× SDS sample buffer.
11. For regular 7.5% gel, SDS-PAGE takes around 1 h at constant 150 V.
12. For Phos-tag-containing gel, SDS-PAGE takes around 2 h at constant 150 V.

Note: Heat is generated during this process. To reduce it, we put the gel tank in an ice-containing tray during SDS-PAGE. The size of GST-ATG8-family proteins is ∼37 kDa. Please keep checking the protein marker and stop gel running when the 25 kDa protein marker reaches the bottom of the gel.

13. Shake the Phos-tag gel in 1 mM EDTA-containing transfer buffer for 20 min.

Note: The manganese ion (Mn^{2+}) affects protein transfer onto PVDF membrane. EDTA can help reduce the manganese ion-caused effect on protein transfer.

14. Wash the Phos-tag gel with transfer buffer on shaker for 10 min.
15. For gel transfer, four transfer buffer-soaked filter papers and one methanol-treated PVDF membrane per gel are needed.
16. From bottom to top, place two filter papers, PVDF membrane, protein gel and two filter papers in the Semi-Dry Blotting System (or similar transfer equipment).

Note: Rinse the gels with transfer buffer before putting it on top of the PVDF membrane.

17. Pour 3–5 mL transfer buffer on the top and get rid of bubbles using a roller.
18. Load the Semi-Dry Blotting System cover. Operate the transfer for 1 h 30 min at constant 0.3 mA/gel.
19. Block the PVDF membranes with 5% non-fat milk for 30 min on shaker.
20. Wash the PVDF membranes with 1× TBST buffer.
21. Prepare the primary antibody solutions (i.e., GST, Flag) using 5% non-fat milk with suitable dilution.
22. Incubate the PVDF membranes with the primary antibody solutions on shaker in cold room for 12–16 h.
23. Next day, wash the PVDF membranes with 1× TBST buffer on shaker for three times, 10 min/time at 20–25°C.
24. Prepare secondary antibody solutions using 5% non-fat milk with suitable dilutions.
25. Incubate the PVDF membranes with the secondary antibody solutions on shaker at 20–25°C for 1 h.
26. Wash the PVDF membranes with 1× TBST buffer on shaker for three times, 10 min/time at 20–25°C.

Image processing

Timing: 15 min

27. Turn on a ChemiDoc instrument (or similar equipment).
28. Prepare the ECL solution mixture.

Figure 2. Analysis of MAP4K2-induced LC3A and LC3B phosphorylation using Phos-tag gel

(A) The S87 site is conserved between human LC3A and LC3B. Sequence alignment is performed for the amino acids surrounding LC3A S87 site and LC3B S87 site.

(B) MAP4K2 phosphorylates LC3A at multiple sites including S87. MAP4K2-mediated LC3A phosphorylation induces the band shift of LC3A protein in Phos-tag gel, which can be largely but not completely reversed by its S87A mutation.

(C) MAP4K2 phosphorylates LC3B at multiple sites including S87. MAP4K2-mediated LC3B phosphorylation induces the band shift of LC3B protein in Phos-tag gel, which can be significantly but not completely reversed by its S87A mutation.

a. Mix Solution A and Solution B at 1:1 ratio.

29. Incubate the PVDF membranes with the ECL solution mixture for 1 min.
30. Place the PVDF membranes on a plastic wrap and cover them with another plastic wrap.
31. Collect the chemiluminescent signals using the ChemiDoc instrument.

EXPECTED OUTCOMES

MAP4K2 is known to phosphorylate multiple sites of LC3A and LC3B proteins.[1] Among them, the S87 site is the major phosphorylation site on LC3A, which is also conserved in LC3B (Figure 2A). Through *in vitro* kinase assay, the MAP4K2-mediated phosphorylation will induce the band shift for GST-LC3A (Figure 2B) and GST-LC3B (Figure 2C). Moreover, mutating S87 to Ala will reduce the band shift for GST-LC3A (Figure 2B) and GST-LC3B (Figure 2C).

LIMITATIONS

First, the S87A mutation cannot fully reverse the MAP4K2-induced band shift for LC3A and LC3B in Phos-tag gel, suggesting the existence of additional phosphorylation sites. Therefore, further mutagenesis and site-mapping work is needed to reveal the rest phosphorylation site(s) for these two LC3 proteins. Second, conditions, such as Phos-tag concentration, Mn^{2+} concentration, and SDS-PAGE running time, need to be optimized for the use of Phos-tag gel. Third, based on our experience, not all proteins can be analyzed for their phosphorylation using Phos-tag gel.

TROUBLESHOOTING

Problem 1

Substrate mobility shift is not observed in Phos-tag gel (related to GST-ATG8-family protein preparation, SFB-kinase preparation, and preparation of SDS-PAGE gel in "before you begin" section; *in vitro* kinase assay in "step-by-step method details" section).

Potential solution

- Adjust the ratio of kinase and substrate for *in vitro* kinase assay. Increasing the amount of kinase or reducing that of substrate in the kinase assay will improve the mobility shift.

- Before kinase purification, kinase activity can be increased by treating cells with the unique stimuli for kinase.[2,3] For example, cells transfected with SFB-MAP4K2 can be treated glucose starvation for 12–16 h[1] and then subjected to SFB-MAP4K2 purification using S-protein beads.
- For Phos-tag gel preparation, the concentration of Mn^{2+} can affect the mobility shift of phosphorylated protein. Testing its concentration from 50 μM to 150 μM is suggested for optimizing the condition.
- Cleave the GST tag to reduce the protein molecular weight may allow monitoring of more subtle difference in phosphorylation.

Problem 2

Protein band shape is changed in Phos-tag gel (related to Western blotting in "step-by-step method details" section).

Potential solution

- Use ice-cold transfer buffer.
- Use low voltage (e.g., 100 v) for SDS-PAGE to further reduce the heat.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wenqi Wang (wenqiw6@uci.edu).

Technical contact

Further technical questions and information should directed to and will be fulfilled by the technical contact, Gayoung Seo (gayoungs@uci.edu).

Materials availability

There are no newly generated materials associated with this protocol.

Data and code availability

There are no datasets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

W.W. and G.S. conceived the study. G.S. performed all the experiments. G.S. and W.W. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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