Protocol

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Highlights

- Protocol for activity of Hippo pathway components in cultured cells
- Detection of YAP phosphorylation with a phos-tag technique
- Measuring LATS kinase activity with an in vitro kinase assay
- Determining YAP or TEAD subcellular localization with an immunofluorescence technique
Protocol

Protocols for measuring phosphorylation, subcellular localization, and kinase activity of Hippo pathway components YAP and LATS in cultured cells

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SUMMARY

The Hippo pathway plays critical roles in cell growth, differentiation, development, tissue homeostasis, and cancer. Here, we describe protocols to measure phosphorylation, subcellular localization, and kinase activity for core Hippo pathway components YAP and LATS. The phos-tag technology is particularly useful to quantitatively detect protein phosphorylation even without a phosphospecific antibody. We use HEK293A cells as an example, but the protocols can be applied to other cell lines and tissues.

For complete details on the use and execution of this profile, please refer to Luo et al. (2020).

BEFORE YOU BEGIN

Note: For detection of YAP phosphorylation (Part I), follow steps 1–4 in BYB and steps 1–12 in Step by Step; For detection of LATS kinase activity (Part II), follow steps 1–2 of BYB and steps 13–23 in Step by Step; For detection of YAP or TEAD subcellular localization (Part III), follow steps 1–2 of BYB and 24–36 in Step by Step.

Prepare materials and cells

© Timing: 3 days

1. Start a new HEK293A cell culture. Resuscitate low passage HEK293A cells and seed into a 100 mm Cell Culture Dish. Grow cells in a standard culture incubator and passage the cells at the log phase to always keep cells healthy and > 90% viable.

Note: Low passage cells are used to ensure cell quality. It is not recommended to use cells with altered morphology or growth rate, or cells that have been passaged less than 2 times or too many times from frozen stock.
**Note:** Cells should be tested for and free of mycoplasma contamination. Unlike bacterial or fungal contaminations, mycoplasma infection cannot be detected by visual inspection and may not noticeably affect cell growth. However, mycoplasma contaminations have been reported to alter DNA, RNA and protein synthesis (Drexler and Uphoff, 2002). Short tandem repeat (STR) analysis should also be performed to ensure the quality and integrity of human cell lines.

**Note:** All cell culture plates or dishes are incubated at 37°C with humidified 5% CO₂ in a standard culture incubator (Thermo Fisher Scientific).

2. Prepare DMEM with 10% fetal bovine serum (FBS) and 1× antibiotic solution for cell culture of HEK293A beforehand. The cell culture medium are stored at 4°C and warmed to 37°C before use. Ensure there are sufficient solutions and reagents needed for this protocol. Especially 4× SDS-PAGE Sample Buffer, 10× SDS Running Buffer and 10× Transfer Buffer (Methanol-free) need to be prepared in advance.

△ CRITICAL: When preparing the above various buffers, high purity compounds within the validity period are strongly recommended, such as AR grade Tris base, glycine and SDS, etc. Expired or impure compounds will affect the results of the Phos-tag gel. For example, if Tris-HCl buffer is stored at 15°C–25°C for more than a year, white flocculent precipitation will occur in the buffer and affect the pH of the buffer.

**Note:** It is better to prepare fresh blocking buffer (5% milk) and antibody diluent (5% BSA) since the milk and BSA in the buffer will support microbe growth, which can have an adverse impact on the results of the immunoblot analysis.

3. Because a lot of heat will be generated during the transfer process, the 1× transfer buffer (containing methanol) must be pre-cooled at 4°C at least 24 h in advance.

4. The formula for the preparation of 6% Phos-tag gels or 8% Regular gels is listed in the materials and equipment.
   a. For separating gels: 10 mL reaction mixture for two gels.
      i. Try to pour ddH₂O into the two glass sheets’ gaps firstly to check if there is any leakage.
      ii. Add 10% APS (ammonium persulfate) and TEMED last. After mixing, separating gel mixture should be carefully added between the two glass sheets.
      iii. Add 1 mL of isopropanol at the top of the gel mixture to ensure uniform gel polymerization.
   b. For stacking gel: 4 mL reaction mixture for two gels.
      i. The comb (15-well, 1.0 mm) should be gently inserted to avoid bubbles following the addition of the stacking gel solution.
      ii. After polymerization, the comb should be carefully pulled out to avoid bending of the gel stripes between lanes.

**Note:** It is recommended to prepare the electrophoresis gels beforehand. To save time, for Regular gels, 4–8 pieces can be prepared at the same time and can be stored at 4°C with humidity for 2–3 weeks. However, Phos-tag gels can only be stored at 4°C for around 3 days. It is recommended to use freshly made Phos-tag gels. In addition, the polymerization time of Phos-tag gel is slightly longer than Regular gel.

**Note:** The Mini-PROTEAN® Tetra Cell Casting Module (Bio-Rad, cat#1658016) is used to prepare the Phos-tag or Regular gels. The Mini-PROTEAN® Tetra electrophoresis and blotting system (Bio-Rad, cat#1703989) are used to perform immunoblot analysis in this protocol.
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rabbit monoclonal anti-LATS1 (C6685) | Cell Signaling Technology | Cat#3477 |
| Rabbit monoclonal anti-pLATS1(T1079) (D57D3) | Cell Signaling Technology | Cat#14074 |
| Rabbit monoclonal anti-YAP (D8H1X) | Cell Signaling Technology | Cat#4911 |
| Rabbit polyclonal anti-pYAP(S127) | Cell Signaling Technology | Cat#49332 |
| Rabbit polyclonal anti-MST1 | Cell Signaling Technology | Cat#3682 |
| Rabbit monoclonal anti-pMST1(T183) (E7U1D) | Cell Signaling Technology | Cat#2729 |
| Rabbit monoclonal anti-pGAPDH | Santa Cruz Biotechnology | sc-25778 |
| Anti-rabbit IgG, HRP-linked Antibody | Cell Signaling Technology | Cat#7074 |
| Anti-mouse IgG, HRP-linked Antibody | Cell Signaling Technology | Cat#7076 |
| Normal Rabbit IgG (1 mg/mL) | Cell Signaling Technology | Cat#2729 |
| Mouse monoclonal anti-YAP/TAZ (63.7) | Santa Cruz Biotechnology | sc-101199 |
| Rabbit monoclonal anti-Pan-TEAD (D3F7L) | Cell Signaling Technology | Cat#3295 |
| Goat anti-Rabbit IgG, Alexa Fluor 488 | Thermo Fisher Scientific | Cat#A-11008 |
| Goat anti-mouse IgG, Alexa Fluor 555 | Thermo Fisher Scientific | Cat#A-21422 |
| Chemicals, peptides, and recombinant proteins |        |            |
| Dulbecco's Modified Eagle Medium (DMEM) | Gibco | Cat#C11885500BT |
| Fetal Bovine Serum (FBS) | Gibco | Cat#10099141 |
| Penicillin-Streptomycin 100× solution | HyClone | Cat#SV30010 |
| NaCl (Sodium Chloride) | Sigma-Aldrich | Cat#S7653 |
| 1× PBS | Gibco | Cat#C10010500BT |
| 30% Acrylamide | Solarbio Life Sciences | Cat#A1010 |
| Tris-HCl (1 M, pH 6.8) | Solarbio Life Sciences | Cat#T1020 |
| Tris-HCl (1 M, pH 8.8) | Solarbio Life Sciences | Cat#T1010 |
| Tris-HCl (1 M, pH 7.5) | Solarbio Life Sciences | Cat#T1140 |
| Tris base | Sigma-Aldrich | Cat#V9001483 |
| Glycine | Sigma-Aldrich | Cat#V900144 |
| Ammonium Persulfate (APS) | Sigma-Aldrich | Cat#A3678 |
| Sodium dodecyl sulfate (SDS) | VWR | Cat#0227-55G |
| Bromophenol blue | Sigma-Aldrich | Cat#114391 |
| Glycerol | Sigma-Aldrich | Cat#V900122 |
| Beta-mercaptoethanol | Sigma-Aldrich | Cat#M6250 |
| MnCl2·4H2O | Sigma-Aldrich | Cat#M3634 |
| Phos-tag (Critical Reagents) | Wako Pure Chemicals Industrials, Ltd | Cat#304-93521 |
| TEMED | Solarbio Life Sciences | Cat#T8090 |
| Methanol | VWR Chemicals | Cat#VWR85650.360 |
| Skim Milk | Bio-Rad Laboratories | Cat#1706404 |
| Albumin bovine serum (BSA) | Sigma-Aldrich | Cat#V900193 |
| Pre-stained Protein Ladder | Thermo Fisher Scientific | Cat#26616 |
| Immobilon Crescendo Western HRP substrate | Merck Millipore | Cat#WBSTR0500 |
| Triton X-100 | Sigma-Aldrich | Cat#X100 |
| Protease/Phosphatase Inhibitor Cocktail (100×) | Cell Signaling Technology | Cat#S872 |
| ATP (10 mM) | Cell Signaling Technology | Cat#9804 |
| Protein Kinases Buffer (10×) | Cell Signaling Technology | Cat#9802 |
| GST-YAP | Homemade | N/A |
| 4% Paraformaldehyde | Fisher Scientific | Cat#S0-259-98 |
| Goat Serum | Sigma-Aldrich | Cat#G9203 |
| ProLong® Gold Antifade Reagent with DAPI | Invitrogen | Cat#P36931 |
| Poly-L-ornithine solution (0.1 mg/mL) | Sigma-Aldrich | Cat#P4957 |
| DAPI Solution (1 mg/mL) | Thermo Fisher Scientific | Cat#62248 |

(Continued on next page)
### MATERIALS AND EQUIPMENT

#### HEK293A cell medium

| Reagent                  | Amount   | Final concentration |
|--------------------------|----------|---------------------|
| DMEM 1×                  | 450 mL   | N/A                 |
| FBS                      | 50 mL    | 10%                 |
| Antibiotic solution, 100×| 5 mL     | 1×                  |

Store at 4°C for up to 3 months. Pre-warm to 37°C before use.

#### 4× SDS-PAGE Sample Buffer

| Reagent                  | Amount   | Final concentration |
|--------------------------|----------|---------------------|
| Tris-HCl (1 M, pH 6.8)   | 10 mL    | 200 mM              |
| SDS                      | 4 g      | 8%                  |
| Bromophenol blue         | 50 mg    | 0.1%                |
| Glycerol                 | 20 mL    | 40%                 |
| Beta-mercaptoethanol     | 10 mL    | 20%                 |
| ddH₂O                    | Add up to 50 mL | N/A |

**Total** 50 mL  
Store at 15°C–25°C for up to 1 year.

#### 10× SDS Running Buffer

| Reagent | Amount   | Final concentration |
|---------|----------|---------------------|
| Tris base | 300 g    | 250 mM              |
| Glycine | 1440 g   | 1.92 M              |
| SDS     | 100 g    | 1%                  |
| ddH₂O   | Add up to 10 L | N/A |

**Total** 10 L  
After fully dissolved, store at 15°C–25°C for up to 6 months.
### 10× Transfer Buffer (Methanol-free)

| Reagent          | Amount       | Final concentration |
|------------------|--------------|---------------------|
| Tris base        | 378.8 g      | 310 mM              |
| Glycine          | 1800 g       | 2.4 M               |
| ddH₂O            | Add up to 10 L | N/A                |
| **Total**        | 10 L         | N/A                 |

After fully dissolved, store at 4°C for up to 6 months.

### 1 × Transfer Buffer (Containing Methanol)

| Reagent          | Amount   | Final concentration |
|------------------|----------|---------------------|
| 10× Transfer Buffer | 0.8 L   | 1×                  |
| ddH₂O            | 7.2 L    | N/A                 |
| Methanol         | 2 L      | 20%                 |
| **Total**        | 10 L     | N/A                 |

Store at 4°C for up to 6 months.

### Formula for the preparation of 6% Phos-tag gels (for 2 gels)

| Reagent                          | Amount for separating gel | Amount for stacking gel |
|----------------------------------|---------------------------|-------------------------|
| ddH₂O                            | 5.5 mL                    | 2.7 mL                  |
| 30% Acrylamide                   | 2 mL                      | 0.67 mL                 |
| Tris-HCl (1.5 M, pH 8.8)         | 2.5 mL                    | N/A                     |
| Tris-HCl (1.0 M, pH 6.8)         | N/A                       | 0.5 mL                  |
| 10% SDS                          | 100 μL                    | 40 μL                   |
| 10 mM MnCl₂                      | 50 μL                     | N/A                     |
| 5 mM Phos-tag                    | 30 μL                     | N/A                     |
| 10% APS                          | 60 μL                     | 40 μL                   |
| TEMED                            | 20 μL                     | 4 μL                    |
| **Total**                        | 10 mL                     | 4 mL                    |

After polymerization, stored at 4°C with humidity for 3 days.

### Formula for the preparation of 8% Regular gels (for 4 gels)

| Reagent                          | Amount for separating gel | Amount for stacking gel |
|----------------------------------|---------------------------|-------------------------|
| ddH₂O                            | 9.3 mL                    | 5.5 mL                  |
| 30% Acrylamide                   | 5.3 mL                    | 1.3 mL                  |
| Tris-HCl (1.5 M, pH 8.8)         | 5 mL                      | N/A                     |
| Tris-HCl (1.0 M, pH 6.8)         | N/A                       | 1 mL                    |
| 10% SDS                          | 200 μL                    | 80 μL                   |
| 10% APS                          | 200 μL                    | 80 μL                   |
| TEMED                            | 12 μL                     | 8 μL                    |
| **Total**                        | 20 mL                     | 8 mL                    |

After polymerization, stored at 4°C with humidity for 2–3 weeks.

### 10× Tris-buffered saline and tween 20 (TBST) (pH7.6)

| Reagent          | Amount       | Final concentration |
|------------------|--------------|---------------------|
| Tris Base        | 24.2 g       | 200 mM              |
| NaCl             | 80 g         | 1.37 M              |
| Tween 20         | 5 mL         | 0.5%                |
| ddH₂O            | Add up to 1 L | N/A                 |
| **Total**        | 1 L          |                     |

Store at 15°C–25°C for up to 1 year. Use ddH₂O to dilute 10 times to get 1× TBST (pH7.6).
### 10% APS (Ammonium Persulfate)

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

After fully dissolved, filter using 0.45 μM filter unit. Store at 4°C for up to 1 year and protect from light.

### 10% SDS (Sodium Dodecyl Sulfate)

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

After fully dissolved (avoiding bubbles), store at 15°C–25°C for up to 1 year.

### 100 mM MnCl₂

| Reagent   | Amount | Final concentration |
|-----------|--------|---------------------|
| MnCl₂·4H₂O | 0.198 g | 100 mM              |
| ddH₂O     | Add up to 10 mL | N/A               |

Store at 15°C–25°C for up to 1 year.

### 5 mM Phos-tag

To prepare phos-tag solution, add 100 μL of methanol to 10 mg of phos-tag powder, dissolve the powder by vigorous vortex, and add 3.2 mL of ddH₂O and mix well. Aliquot the solution into tubes wrapped with aluminum foil to protect from light and store at 4°C for up to 1 year. Generally, 30 μL of the phos-tag solution is sufficient for two mini gels.

### 5% BSA (Bovine Serum Albumin)

| Reagent   | Amount | Final concentration |
|-----------|--------|---------------------|
| BSA       | 5 g    | 5%                  |
| 1x TBST   | Add up to 100 mL | N/A               |

After fully dissolved, store at –20°C for up to 3 months.

### 5% milk

| Reagent   | Amount | Final concentration |
|-----------|--------|---------------------|
| Skim milk | 5 g    | 5%                  |
| 1x TBST   | Add up to 100 mL | N/A               |

Prepared for immediate use, or stored at 4°C for no more than 1 day.

### Mild Lysis Buffer (MLB)

| Reagent   | Amount | Final concentration |
|-----------|--------|---------------------|
| Tris-HCl (1 M, pH 7.5) | 25 mL | 50 mM               |
| NaCl (1 M)      | 75 mL | 150 mM              |
| Triton X-100   | 2.5 mL | 0.5%                |
| ddH₂O         | 397.5 mL | N/A                |
| Total         | 500 mL | N/A                |

Store at 4°C for up to 6 months. Take out just before use and keep on the ice.
CRITICAL: Reagents used may have potential hazards, such as acrylamide, SDS, beta-mercaptoethanol, TEMED, methanol and paraformaldehyde. Their harmful or toxic include one or more of the following: skin/eye corrosion/irritation, allergic skin reaction, genetic defects, carcinogenicity, etc. When handling these reagents, preventive measures include protective gloves/protective clothing/eye protection/face protection. Experiments should be performed in a well-ventilated area, and wash face, hands and any exposed skin thoroughly after handling.

Alternatives: In addition to Cell Signaling Technology, some Hippo pathway antibodies can also be purchased from the following companies, such as Santa Cruz Biotechnology, Abcam, and Bethyl Laboratories. However, we recommend using the antibodies from the sources listed in the reagent table because their quality has been verified through many experiments.

**STEP-BY-STEP METHOD DETAILS**

**Part I: Detection of YAP phosphorylation**

*Note:* This protocol is modified from Chen et al. (2019).

**Seeding HEK293A cells**

- **Timing:** 1 h

In this section, all cell culture steps are to be performed under “aseptic conditions” in a cell culture hood.

1. Once HEK293A cells reach 80%–90% confluency, they are ready for passage.
a. Aspirate old culture media from the 100 cm culture dish and gently rinse the cells with ~3.0 mL of sterile 1× PBS.
b. Add 2 mL of pre-warmed 0.25% Trypsin-EDTA directly to the cells and incubate at 37°C for 1–2 min until cells become shrunk and round and have detached from the bottom of the dish. During the digestion process, you can tap the edge of the culture dish with your hand.
c. Add 3 mL of pre-warmed DMEM with FBS to neutralize Trypsin-EDTA and mix the cells by pipetting up and down repeatedly until achieving a single-cell suspension, and then transfer the approximately 5 mL cell suspension to a 15 mL conical centrifuge tube.
d. Centrifuge the tube at 3000 x g for 3 min, discard the supernatant and resuspend the cell pellet with an appropriate amount of pre-warmed DMEM with FBS and mix well by pipetting up and down several times.
e. Count cells using the automated cell counter (Countstar) and plate the cells into 6-well plates at a density of 5 x 10^5 to 8 x 10^5 cells/well, depending on the experimental needs. Add pre-warmed DMEM with FBS to make a final volume of 2 mL/well. Culture the cells in a standard incubator for 24 h.

**Note:** The amount of cells can be scaled down to 12 or 24 well plates to meet experimental needs.

△ **CRITICAL:** Cell density will affect the phosphorylation of Hippo pathway components, including YAP, TAZ, LATS and MST (Meng et al., 2016). Seed appropriate cell numbers into each well to ensure that the cells reach the desired confluence the next day.

△ **CRITICAL:** It is recommended to overexpress MST1 by transient transfection because the phosphorylation signal of endogenous MST1 in HEK293A cells is a little weak and difficult to be detected.

**Collect cell lysis for immunoblot analysis**

△ **Timing:** 0.5 h for collect cell lysate, 2 days for immunoblot analysis

In this section, the cell lysate will be subjected to immunoblot analysis to detect the phosphorylation for Hippo pathway components. This experiment is described separately according to the two different types of gels, i.e., Phos-tag gel for YAP phosphorylation analyses and regular gel for pYAP(S127), pLATS1 (T1079) and pMST1(T183) analyses.

2. Collect cell lysate: Aspirate old culture media as completely as possible. Then harvest cells by directly adding 300–1000 μL 1 x SDS-PAGE Sample Buffer per well in 6-well plates, and shake the plate thoroughly using a plate shaker at 150 x g for at least 10 min. Transfer the sample to 1.5 mL microcentrifuge tubes and heat at 95°C–101°C for 5 min. Centrifuge at 16,000 x g for 5 min at 15°C–25°C and store at −20°C for subsequent immunoblot analysis.

**Note:** You can treat the cells with designed stimulation before collecting the cells, such as heat shock, serum starvation, small molecule compounds, osmotic stress, etc.

**Note:** There is no need to add Protease/Phosphatase Inhibitor Cocktail to the sample buffer since all protein is denatured quickly. Cell lysates prepared in the 1 x SDS-PAGE Sample Buffer can be stored at −20°C for three months.

**Note:** No need to determine the protein concentrations using a commercial Bradford assay or BCA Protein Assay Kit as long as each culture well has similar amount of cells. Their concentrations in different samples can be normalized by adjusting the volume of the Sample Buffer. In general, about 100 μL of Sample Buffer is used for every 2 x 10^5 cells in the well. For
example, when the HEK293A cells reach 100% confluence in a 6-well plate, the number of cells is approximately $1.5 \times 10^6$ and 800 µL of Sample Buffer should be added. If the cell confluence only reaches 50% in the treatment well, add only 400 µL of Sample Buffer accordingly.

3. Loading sample: Take out the comb carefully and aspirate any remaining liquid in the wells before loading the samples. Load 10–15 µL of sample per well.

Note: Protein ladder should be loaded on the leftmost and rightmost side of the sample well, and judge your sample loading order by the color shade of protein ladder. Dilute the pre-stained protein ladder from Thermo Fisher Scientific using 1× SDS-PAGE Sample Buffer (250 µl protein ladder diluted in 1 ml Sample Buffer), and load the same volume of blank sample buffer to any empty wells to ensure uniform electrophoresis.

4. Electrophoresis: Start electrophoresis at 80 V for 30 min, until all the samples enter the separating gel.

For Phos-tag gel, then run at 100 V for about 2.5 h until the 55 kDa marker in the pre-stained protein ladder is around 1.5 cm from the bottom of the gel (YAP protein is above the 70 kDa marker).

For Regular gel, then run at 120 V for about 1.5 h until the bromophenol blue indicator line is around 0.5 cm from the bottom of the gel.

5. Transferring: The PVDF membrane with a 0.45 µm pore size is activated by soaking in methanol for 2 min. Assemble the wet transferring system, and transfer for 2 h (Phos-tag gel) or 1 h (Regular gel) at 4°C in the cold room or refrigerator. Set constant current at 370 mA for one Trans-Blot tank, or 740 mA for two Trans-Blot tanks.

Note: The filter paper used for blotting transfer should be chromatography paper. The thickness of the filter paper on one side of Gel Holder Cassettes should be 0.7–0.8 mm. Therefore, use 2 sheets of filter paper with a thickness of 0.4 mm on one side. If use commercially available filter paper with a thickness of 0.8 mm, one sheet on each side is sufficient.

△ CRITICAL: It takes more time to transfer YAP protein from phos-tag gel to PVDF membrane than that of regular gel. Therefore, in order to avoid overheating of the transfer buffer, it is strongly recommended to change the Ice Cooling Unit after 1 h’s phos-tag gel transferring.

6. Wash the whole PVDF membrane one time with 1× TBST for 5 min.
7. Block with 5% milk in TBST for 1 h at 15°C–25°C with gentle rocking.
8. Dilute the primary antibody in 5% BSA in TBST. Incubate and rock the membrane with primary antibody around 10–15 h at 4°C.

Note: The primary antibody should be diluted in 5% BSA at concentrations recommended by the manufacturer. Details as follows: YAP, 1:1,000; LATS1, 1:2000; MST1, 1:2000; pYAP (S127), 1:1000; pLATS1 (T1079), 1:1000; pMST1 (T183), 1:500; GAPDH, 1:4000. The primary antibody solution can be used multiple times depending on the quality of the antibody. For a short term (within a week), the antibody solution can be stored at 4°C to avoid freeze-thaw cycles. For a long time (more than a week), we suggest to store the antibody solution at −20°C.

△ CRITICAL: The antibody against YAP protein is used as primary antibody in the Phos-tag gel analysis. Please do not use the phosphoantibodies of pYAP(S127).
9. The next morning, pipette out the primary antibody and save for future use (the antibody can be reused for 5 times usually). Wash the blot 3 times with 1× TBST with rocking for 10 min each.
10. Incubate with HRP-linked secondary antibody (diluted in 5% milk in TBST at 1:5000) for 1 h at 15°C–25°C.

**Note:** Be sure to use a secondary antibody that can recognize the source of the specific primary antibody (most are from rabbit or mouse).

11. Wash the blot 4 times with 1× TBST for 15 min each with rocking.
12. Incubate the membrane with the Western HRP substrate for 1 min at 15°C–25°C. Remove the membrane from the working reagent and absorb any excess liquid or bubbles with Kimwipe or filter paper, place the membrane between two transparent plastic sheets or wrap the membrane with Saran wrap, expose to autoradiography film in an X-ray film cassette, develop the film with a film developer in a dark room.

**Note:** The exposure time varies for different target proteins and antibodies. A good starting point is an exposure time of 1 min. Avoid over exposure time, which will saturate the signal band and conceal the changing trend of the target proteins.

**Part II: detection of LATS kinase activity**

*Seeding HEK293A cells for immunoprecipitation (IP)*

© **Timing:** 1 h

13. Once HEK293A cells reach 80%–90% confluency, they are ready for passage.

**Note:** This protocol certainly does not only apply to 6 well plates. To ensure that sufficient amount of LATS1 is recovered for subsequent IP experiments, it can be scaled up in 100 mm culture dishes to meet experimental needs.

⚠ **CRITICAL:** Cell density will affect the Hippo pathway, including the localization of YAP and activity of LATS1 kinase (Zhao et al., 2007). Seed appropriate cell numbers into 6 well plates or dishes to make sure that the cells reach the desired confluence next day.

**Alternatives:** 24 h after seeding the cells, expression plasmids or siRNAs transfection may be performed if the expression of a gene needs to be modulated. The transfected HEK293A cells can be reseeded.

*Cell lysis, LATS immunoprecipitation and kinase assay*

© **Timing:** around 5 h

14. Prewash the Magnetic Beads: Thoroughly vortex (max speed, 30 s) the commercial magnetic beads tube, then pipette an appropriate amount of beads (10 μL/Sample) into a new 1.5 mL microcentrifuge tube, wash the beads 3 times with mild lysis buffer (MLB) and final resuspend with MLB with protease/phosphatase inhibitor cocktails (20 μL/Sample).

**Note:** Consider the amount of loss during operation, prepare the magnetic beads for (n + 2) samples.

**Note:** Detailed wash method: add 1 mL of wash buffer to the tube containing an appropriate amount of beads, vortex thoroughly and place the tube into a magnetic stand for 2 min to
collect the beads against the side of the tube; remove the supernatant; take out the tube from the magnetic stand, repeated the above washing for total 3 times.

15. For HEK293A cells in the 6 well plates or dishes, gently aspirate old media, wash twice with cold PBS gently.

**Note:** You can treat the cells with various signals before collecting the cells, such as heat shock, serum starvation, small molecule compounds, osmotic stress.

△ CRITICAL: HEK293A cells detach from the surface of plate very easily. Thus, all rinsing steps should be performed carefully and gently, as to not cause HEK293A detachment. LATS activity can be rapidly perturbed by many environmental factors, thus ice-cold PBS should be used to wash the cells and harvest should be completed promptly on ice.

16. Add 1 mL MLB with protease/phosphatase inhibitor cocktails directly onto the cells for each sample, shake the plates or dishes at 4°C for 10 min, and then transfer the cell-containing solution to 1.5 mL microcentrifuge tubes.

17. Spin at 16,000×g, 4°C for 10 min, to separate all the cell debris from the supernatant, and transfer the lysis supernatant to a new 1.5 mL microcentrifuge tubes, which need to be maintained on ice.

18. Add LATS1 antibody (CST, 1: 1000) and pre-washed magnetic beads resuspended in MLB (20 uL/sample) (from step 14) together into the lysis samples prepared in step 17, invert gently to mix at 4°C for 2 h. Include a negative control with the normal rabbit IgG (the LATS1 antibody is rabbit IgG). Photos of sample tubes with an inverted mixing at 4°C can be seen in Figure 1A.

△ CRITICAL: In conventional IP experiments, after adding specific antibodies, the samples usually be incubated around 10–15 h at 4°C with mixing. But in the kinase assay experiment, to ensure the activity of LATS1 kinase, the incubation time should not exceed 2 h.

**Note:** The concentration of Normal Rabbit IgG is 1μg/μL, which is higher than the concentration of LATS1 antibodies from the CST company (around 0.2 μg/μL). Thus, the IgG control needs to be diluted with MLB to the same concentration before use.

19. After 2 h’s incubation, collect the beads with a magnetic stand, remove the supernatant, then wash 5 times with MLB and subsequently 1 time with 1× Protein Kinases Buffer. A photo of sample tubes on a magnetic stand to collect the beads against the side of the tube is shown in Figure 1B.

![Figure 1A](image1A.png)

**Figure 1A.** Sample tubes were inverted gently by a rotator to mix the lysis, antibody, and magnetic beads at 4°C.

![Figure 1B](image1B.png)

**Figure 1B.** Magnetic beads were collected against the side of the tube by a magnetic stand.
Critical: The remaining reagent components in the tube may affect the kinase reaction, so it must be thoroughly washed with MLB at least 5 times, and the 1× Protein Kinases Buffer should be used for the last washing.

**Note:** Detailed wash method: add 1 mL of wash buffer into each tube, invert the tube several times and gently shake at 4°C for 5 min, then place the tube back into a magnetic stand for 2 min to collect the beads against the side of the tube; remove the supernatant; repeat above washing steps.

20. Add 40 μL of kinase reaction buffer with substrate GST-YAP per sample directly to the above-washed beads in step 19, mix gently by finger tap, and incubate on a heat shaker with 300 rpm at 30°C for 30 min.

**Critical:** To ensure the reaction quality, it’s better to make the reaction buffer mix just before use and samples can be manually mixed by a finger tap every 10 minutes during the incubation.

21. After 30 min at 30°C, add 13.3 μL of 4× SDS-PAGE sample buffer to the tube, vortex to mix completely and then heat the samples at 100°C for 5 min.

22. Spin down the magnetic beads/samples at 16,000 g, 15°C–25°C for 10 min, then magnetically separate the beads and transfer the supernatant to a new microcentrifuge tube and saved at −20°C for immunoblot analysis.

Pause Point: Lysates prepared for immunoblot analysis can be stored at −20°C for several weeks.

**Detection of YAP phosphorylation using immunoblot analysis**

**© Timing:** 2 days

23. Please refer to the corresponding step 2–12 in Part I.

**Part III: detection of YAP or TEAD subcellular localization**

**Note:** This protocol is modified from Lin et al. (2017).

**Seeding HEK293A cells for immunofluorescence (IF)**

**© Timing:** 1.5 h

24. Once HEK293A cells reach 80%–90% confluent growth, prepare and collect cells for seeding. For most information, please refer to the corresponding steps in “Part I”. For the last step, plate cells into 12-well plates on sterile round coverslips at a density of 2 x 10^5 cells/1 mL/well. Culture the cells in a standard incubator around 10–15 h.

**Note:** For Immunofluorescence assay, usually a 24-well plate or a 12-well plate is sufficient to meet experimental needs, and it is not necessary to use a 6-well plate. When seeding the cells into the well, make sure no air bubbles are trapped underneath the coverslips and get an even distribution of cells.

**Note:** The coverslips need to be sterilized by immersing in ethanol or autoclaved. If using ethanol for sterilization, the coverslips need to dry completely by evaporation before use.
**Critical:** Cell density will affect the subcellular localization of YAP/TAZ or TEAD (Meng et al., 2016). Seed appropriate cell numbers into 12 or 24 well plates to make sure that the cells reach the desired confluence the next day.

**Alternatives:** To enhance cell adherence, coverslips may be pre-coated with poly-L-ornithine as follows:

a. Use sterile forceps to carefully place one sterile coverslip into one well of the 12-well plates.
b. Pretreat the coverslip with poly-L-ornithine solution diluted 1:20 at 37°C for 20 min. For one well, dilute 25 μL poly-L-ornithine (0.1 mg/mL) into 500 μL sterile 1× PBS (Final working concentration 0.005 mg/mL).
c. Aspirate the coating solution and wash with PBS for several times. Keep the plates in a tissue culture hood for a period of time to allow the coverslip to dry completely.d. Then seed cells on poly-L-ornithine-coated coverslips.

**Critical:** HEK293A cells are very easy to fall off the coverslip during the permeabilization and wash steps of immunofluorescence. It is strongly recommended to coat the coverslips.

**Immunofluorescence staining**

** Timing:** 2 days

25. Aspirate the old medium in wells, rinse them couple times with PBS.

   **Note:** You can treat the cells with various signals before staining, such as heat shock, serum starvation, small molecule compounds, osmotic stress.

26. Fix cells with 500 μL 4% paraformaldehyde in PBS for 10 min at 15°C–25°C.

   **Note:** The goal of fixation is to maintain the cellular structure as close as possible to the native state. There are several fixation methods suitable for IF, like paraformaldehyde or methanol. You can choose the best one for your target proteins.

27. Wash with PBS 3 times.

28. Permeabilization with 500 μL 0.2% Triton X-100 in PBS for 10 min at 15°C–25°C.

   **Note:** The permeabilization step help antibodies entering the fixed cells. Besides Triton X-100, various other detergents can be used according to your experimental needs, including NP-40, Tween-20 and Saponin.

29. Wash with PBS 3 times.

30. Blocking with 2% BSA+2% Goat Serum in PBS for 30 min at 15°C–25°C.

31. Prepare all the primary antibodies in 2% BSA in PBS, incubated with the samples around 10–15 h at 4°C. For YAP/TAZ (Clone 63.7, mouse source) 1:200 dilution; PAN-TEAD (Clone D3F7L, rabbit source) 1:500 dilution.

   **Note:** For double/triple staining, the primary antibodies can be mixed before being applied to the coverslips, as long as they can be recognized by different secondary antibodies (such as mouse vs rabbit).

32. Remove the primary antibody, and wash with PBS 3 times.

33. Incubate samples with the Goat anti-Mouse IgG or Goat anti-Rabbit IgG second antibody prepared in 2% BSA in PBS (1:1000 dilution) at 15°C–25°C in the dark for 1 h.
**Note:** Use high-quality and cross-adsorbed the Alexa Fluor secondary antibody conjugates.

34. Wash with PBS 3 times.
35. Mount the coverslips at the center of the slide with ProLong® Gold Antifade Reagent with DAPI, and wait for several hours to let the mounting medium solidifies. Draw off excess mountant before they are examined by microscope.

**Alternatives:** The concentration of DAPI that comes with the commercial mountant is very low, resulting in weak DNA staining. One can stain the samples with 1–10 µg/mL DAPI working solution at 15°C–25°C in the dark for 5 minutes before mounting. The DAPI stock solution is usually 1 mg/mL prepared in pure water.

⚠ **CRITICAL:** Because the fluorescence intensity of the sample is easily bleached during confocal photography, a high-quality antifading mountant should be used. Make sure the coverslip with the cell-side is down. Never try to move the coverslip once it falls on the slide or try to squeeze the few air bubbles trapped between the coverslip and the slide, which will damage the structure of the cells.

36. Images were captured with Olympus FV1000 confocal microscopy or microscope available to you. Immunofluorescence data were collected by NIS-Elements Advanced Research 5.11.00 imaging software and the signals from the channels were merged using ImageJ (1.52a) software.

**EXPECTED OUTCOMES**

Phos-tag is a compound that bind phosphate in the presence of Mn²⁺. Phos-tag was developed to detect protein phosphorylation (Kinoshita et al., 2009). The migration rate will be slower for proteins with a higher degree of phosphorylation in Phos-tag gels. It has been well established that there are at least five LATS1 phosphorylation sites in YAP protein (Zhao et al., 2007). Thus, in Phos-tag gel, YAP can be resolved into multiple bands between 70 kDa to 100 kDa, representing YAP with different levels of phosphorylation (Figure 2A). Latrunculin B (LatB) induces actin depolymerization and activates the Hippo pathway. The top band (around 100 kDa) represents YAP with the highest

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**Figure 2. Immunoblot analyses for phosphorylation of Hippo pathway components**

(A) Heat shock induces YAP dephosphorylation and overrides the effect of Latrunculin B (LatB) that stimulates YAP phosphorylation. An example of YAP Phos-tag gel analyses.

(B) Heat shock induces YAP and LATS1 dephosphorylation and has a minor effect on the phosphorylation of GST-MST1. Phosphorylation was determined by phosphospecific antibody in Regular gel analyses.
degree of phosphorylation which has the slowest migration. The bottom band represents non-phosphorylated YAP (around 70 kDa), which has the fastest migration. Conversely, in regular gels the phosphorylated forms are poorly separated from the unphosphorylated form. Therefore, antibodies for pYAP (S127), pLTAS1 (T1079) and pMST1(T183), essentially show a single band for each protein (Figure 2B).

The activity of LATS1 kinase is positively correlated with the degree of YAP phosphorylation if substrate is not limiting (Meng et al., 2016). Thus, we usually directly ascertain LATS1 activity using glutathione S-transferase (GST)-YAP as a substrate. LATS1 immunoprecipitated from heat-shocked HEK293A cells exhibited low activity to phosphorylate YAP on S127 (Figure 3) (Luo et al., 2020).

Once the Hippo pathway is activated, the LATS-dependent phosphorylation of YAP/TAZ results in 14-3-3 binding and cytoplasmic localization (Meng et al., 2016). Previous studies suggested that environmental stress promotes TEAD cytoplasmic translocation via p38 MAPK in a Hippo-independent manner (Lin et al., 2017). As expected, environmental stresses such as osmotic stress, induced cytoplasmic translocation of TEAD and YAP/TAZ (Figure 4).

**LIMITATIONS**

The preparation of 6% Phos-tag gels in this protocol is only suitable for detecting YAP or TAZ in the Hippo pathway, the molecular weight of which is about 70 kDa. For other proteins of interest, researchers should optimize the acrylamide concentration, Phos-tag concentration, gel electrophoresis and transferring time, to ensure that the phosphorylated form of target proteins can be properly separated. In general, the 6% Phos-tag gel should be selected for those proteins of interest with a molecular weight more than 60 kDa; the 8% Phos-tag gel should be selected for those proteins of interest with a molecular weight less than 60 kDa. However, it is hard to separate proteins with a molecular weight greater than 150 kDa by Phos-tag gels. One advantage of Phos-tag gel analysis is that it only needs antibodies against total proteins to detect the phosphorylation of target proteins, which does not require phosphospecific antibodies. However, if the protein of interest has more than one phosphorylation site, the phos-tag gel cannot provide information about its specific phosphorylation sites. Furthermore, one big advantage of phos-tag is that it provides quantitative information regarding the stoichiometry of phosphorylation. It should also be noted, based on our experience, that not all phosphoproteins are well-resolved by Phos-tag gel.

For in vitro kinase assay for MST1 or MAP4K4, recombinant GST-LATS1 or GST-LATS2 should be used as the substrate. For LATS1 or MAP4K4, the amount of cells from one well of a 6-well plate is generally sufficient to provide enough protein for the kinase analysis described above. However, for MST1, this amount should be scaled up in 100 mm culture dishes. Another limitation is that both the IgG heavy chain and the protein (A+G) could alter the shapes of the 55 kDa protein bands due to the similar molecular weight. The protein (A+G) is easy to fall off from the magnetic beads when

![Figure 3. In vitro kinase assay of immunoprecipitated LATS1](image-url)
boiling, so a pH 2.0 buffer can be used to elute the proteins from the magnetic beads. If too much LATS is used in the kinase assay, the substrate GST-YAP might be limiting. Therefore, different amount of immunoprecipitated LATS1 should be tested in the above in vitro kinase assay to ensure that the level of GST-YAP phosphorylation is linearly proportional to the amount of immunoprecipitated LATS1.

The last limitation for YAP or TEAD subcellular localization detection is that it has been optimized for adherent cells. To do immunofluorescence for suspension cells, Thermo Scientific Cytospin 4 Centrifuge needs to be used to attach the suspension cells to slides, which results in forming a uniform monolayer of cells onto an area of a glass slide.

TROUBLESHOOTING

Problem 1
No shift band in the YAP Phos-tag gel. (Steps 2 and 12 in Part I)

Potential solution
If only one band of about 70 kDa can be observed in the YAP Phos-tag gel, or the shift of multiple bands is small, it may be due to the ineffective Phos-tag chemical.

One reason is that Phos-tag has been expired or is not stored protected from light. Another reason may be that the 1X SDS-PAGE sample buffer is contaminated with EDTA or EGTA.

A phos-tag molecule requires at least two Mn²⁺ to bind with phosphate to exert its effect (Kinoshita et al., 2009). Make sure that the sample does not contain EDTA or EGTA, which binds Mn²⁺ thus, interfering the protein mobility shift in Phos-tag gels.

Problem 2
Band dispersion, bending, and tailing. (Steps 2, 3 and 5 in Part I)

Figure 4. Immunofluorescence detects TEAD and YAP/TAZ subcellular localization
HEK293A cells were treated with or without 200 mM NaCl for 6 h and probed with antibodies that recognize all TEAD isoforms (Pan-TEAD) or YAP/TAZ, and followed by fluorescence-labeled secondary antibodies. DAPI stained for DNA. Scale bars, 10 µm.
**Potential solution**
Of note, long-time transferring under constant current will cause protein decomposition and dispersion due to high temperature.

You may perform the electrophoresis in a 4°C environment, and pre-cool the transferring buffer sufficiently before use. It is strongly recommended to change the Ice Cooling Unit in the transfer unit during the transferring process for Phos-tag gel to protect the transfer buffer from overheating.

EDTA, inorganic salts, and surfactants are all potential causes of band dispersion or tailing. These impurities in protein samples can be reduced by TCA precipitation or dialysis.

Additionally, neighboring blank wells can also cause bands to bend, which should be filled with the same volume of 1x SDS-PAGE Sample Buffer as the sample wells.

**Problem 3**
Low resolution of bands in the Phos-tag gel. (Step 4 in BYB)

**Potential solution**
In general, increase the concentration of Phos-tag could enhance the electrophoretic retardation of phosphoprotein. However, increasing the concentration of Phos-tag will not only result in the slowdown of electrophoretic migration of protein, but also cause band smear and waste this expensive reagent. Usually, 30 μL of 5 mM Phos-tag for two single mini gel (final Con. 15 μM) should work for YAP phosphorylation analysis. The Phos-tag concentration can be increased up to 30 μM.

**Problem 4**
Low amount of LATS1 protein was recovered. (Steps 13, 14 and 16 in Part II)

**Potential solution**
Several reasons may reduce the amount of LATS1 protein pulled down.

The LATS1 protein is degraded. Make sure to add Protease/Phosphatase Inhibitor Cocktail into the MLB during step 16.

The number of cells used for LATS1 IP can be scaled up. Although HEK293A cells express a high level of LATS1, LATS1 expression levels in different cell types can vary significantly.

The magnetic beads used in step 14 are not enough. It is noteworthy that the magnetic beads are very easy to aggregate after standing, so they should be thoroughly suspended before each use. Also, never freeze the beads.

**Problem 5**
Low or no activity of recovered LATS1. (Steps 13, 14, 18 and 20 in Part II)

**Potential solution**
If the activity of recovered LATS1 is very low (but the amount of LATS1 protein recovered is OK), it is possible that LATS1 is inactive under the particularly context. Cells with high LATS activity should be collected as a positive control, such as high density cultured and/or serum starved cells.

It is also possible that some problems occurred in the LATS1 pull-down process or kinase reaction system, like insufficient amount of cells, primary antibodies or magnetic beads, poor quality ATP in the reaction buffer.

Make sure that phosphatase inhibitors are present in the lysis buffer.
Avoid incubating the LATS1 antibody, magnetic beads and cell lysate for more than 2 h at 4°C (see step 18). Longer pull-down and incubation time may inactivate LATS1.

**Problem 6**  
No pYAP(S127) band in the western blot exposure result. (Step 23 in Part II)

**Potential solution**  
It is possible that the target strip was cropped from the PVDF membranes. It should be noted that the GST-tag is 220 amino acids (roughly 26 kDa) in size, which, compared to tags such as the HA-tag or the FLAG-tag, is quite large. So, the molecular weight of p(GST-YAP) (S127) is around 110 kDa, instead of the usual 70 kDa position of pYAP(S127).

Also, make sure the pYAP(S127) antibody is good.

**Problem 7**  
Severe non-specific background fluorescence. (Steps 30, 31 and 33 in Part III)

**Potential solution**  
Several reasons may cause non-specific background fluorescence, including low specificity of primary/secondary antibodies, invalid blocking step, and autofluorescence. Setting up multiple negative control groups is very important to ensure the specificity of fluorescence.

Key negative controls include no primary antibody, or no secondary antibody. To further verify the specificity, knockdown or knockout of the target protein should be examined.

**RESOURCE AVAILABILITY**

**Lead contact**  
Further information and requests for resources and reagents should be directed to Min Luo (minluo_scu@163.com).

**Materials availability**  
This study did not generate new unique materials or reagents. HEK293A cells are available from ATCC, many Hippo pathway plasmids are available at Addgene.

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

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**AUTHOR CONTRIBUTIONS**

M.L. and Y.H. drafted the protocol. Y.H. created the graphical abstract. W.X.W. and K.-L.G. developed and revised the protocol.
DECLARATION OF INTERESTS
K.-L.G. is a cofounder of and has an equity interest in Vivace Therapeutics. The other authors declare no competing interests.

REFERENCES
Chen, R., Plouffe, S.W., and Guan, K.L. (2019). Determining the phosphorylation status of hippo components YAP and TAZ using phos-tag. Methods Mol. Biol. 1893, 281–287.

Drexler, H.G., and Uphoff, C.C. (2002). Mycoplasma contamination of cell cultures: incidence, sources, effects, detection, elimination, prevention. Cytotechnology 39, 75–90.

Kinoshita, E., Kinoshita-Kikuta, E., and Koke, T. (2009). Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. Nat. Protoc. 4, 1513–1521.

Lin, K.C., Moroishi, T., Meng, Z., Jeong, H.-S., Plouffe, S.W., Sekido, Y., Han, J., Park, H.W., and Guan, K.-L. (2017). Regulation of Hippo pathway transcription factor TEAD by p38 MAPK-induced cytoplasmic translocation. Nat. Cell Biol 19, 996–1002.

Luo, M., Meng, Z., Moroishi, T., Lin, K.C., Shen, G., Mo, F., Shao, B., Wei, X., Zhang, P., Wei, Y., and Guan, K.L. (2020). Heat stress activates YAP/TAZ to induce the heat shock transcriptome. Nat. Cell Biol 22, 1447–1459.

Meng, Z., Moroishi, T., and Guan, K.L. (2016). Mechanisms of Hippo pathway regulation. Genes Dev. 30, 1–17.

Zhao, B., Wei, X., Li, W., Udan, R.S., Yang, Q., Kim, J., Xie, J., Ikenoue, T., Yu, J., Li, L., et al. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev. 21, 2747–2761.