Screening Kinase-Dependent Phosphorylation of Key Metabolic Reprogramming Regulators

Fatma Necmiye Kaci[1,#], Alessio Lepore[1,#], Salvatore Papa[1], Concetta Bubici[2]

[1] Leeds Institute of Medical Research at St James', Faculty of Medicine and Health, University of Leeds, St. James' University Hospital, Leeds, UK.

[2] Centre for Genome Engineering and Maintenance, Department of Life Sciences, College of Health, Medicine and Life Sciences, Brunel University London, Uxbridge, UK.

[#] These authors contributed equally

Corresponding author:

Concetta Bubici, Email: concetta.bubici@brunel.ac.uk

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Abstract

Aerobic glycolysis has been commonly linked to cell proliferation, especially in cancer cells where it serves to generate sufficient energy and biosynthesis of new cell constituents needed for cell growth and division. The M2 isoform of pyruvate kinase (PKM2) catalyses the last reaction of the glycolytic process. PKM2 promotes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, generating ATP and releasing pyruvate. This rate-limiting reaction relies therefore on the enzymatic activity of PKM2. The switching between the high- and low-activity states of PKM2 is subjected to a combination of allosteric mechanisms and fine-tuned regulation by oncogenes and tumour suppressor genes. These regulatory mechanisms involve primarily post-translational modifications of PKM2. Recent findings suggest that phosphorylation contributes to the regulation of PKM2 activity.
Here, we describe an in-vitro kinase assay we used to assess PKM2 phosphorylation by c-Jun N-terminal kinase (JNK), a master regulator of apoptosis, cell proliferation and differentiation. While the use of phospho-specific antibodies gives information in terms of measuring the effects of a given kinase on its substrate, specific antibodies for newly identified phospho-groups are not readily available. The in-vitro kinase assay allows the immediate measuring of phosphorylation of any substrate of interest. Although there are several options that do not use radioactive materials, we continue to rely on this biochemical method for robust quantitation of results. More interestingly, this protocol can be easily adapted to measure the activity of other kinases by using their specific substrates.

1. Introduction

Highly proliferating cells have the ability to alter their metabolism to sustain an increase in energy demand and biosynthesis of macromolecules required for continuous cell growth, division and survival [1,2]. This cellular ability is best known as metabolic reprogramming (or rewiring) and consists of changes in the expression of critical metabolic enzymes, synthesis of distinct metabolites and stimulation of specific metabolic pathways [1-3]. For instance, most cancer cells convert much of the glucose into lactate irrespective of oxygen availability, allowing highly dividing cells to use intermediary glucose metabolites to generate reducing equivalents (such as NADPH) and macromolecules (such as nucleotides, proteins and lipids) required to suppress basal apoptosis and favouring biomass doubling, respectively [1,2,4]. Before being converted to lactate, the final and rate-limiting step of glycolysis is the conversion of phosphoenolpyruvate (PEP) to pyruvate, which is catalysed by Pyruvate Kinase (PK) [5]. There are four PK isoforms in mammals, PKM1, PKM2, PKL, and PKR. While PKL and PKR are tissue specific, PKM1 and PKM2 are differentially expressed in all tissue, with only PKM2 being expressed in cancer cells [5]. PKM2 is regulated by both allosteric mechanism and covalent post-translational modifications that include primarily phosphorylation [5,6]. Phosphorylation of PKM2 Tyr105 by fibroblast growth factor receptor type 1 (FGFR1) inhibits its catalytic activity, providing a metabolic advantage to tumour cells [7]. ERK2-dependent phosphorylation of PKM2 Ser37 helps to recruit peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (PIN1) for PKM2 cis-trans isomerization that is needed for its translocation to the nucleus [6,8,9]. JNK1-mediated phosphorylation of PKM2 Thr365 enhances PKM2 activity impairing cancer cell survival by promoting apoptosis [6,10]. Of the techniques that have been developed to identify novel kinase phosphosites, the *in-vitro* kinase assay is still the most accurate and used methodology. Here we describe an *in-vitro* kinase assay protocol to asses PKM2 Tyr365 phosphorylation by JNK (**Figure 1**). The advantage of this method is that it can detect direct phosphorylation, it is applicable for

different kinases and diverse substrates, it offers a homogenous and robust assay with minimal false hits.

2. Materials

Prepare all solutions using ultrapure water and use molecular biology grade reagents. Standard solutions should not be stored for longer than three months. For the *in-vitro* kinase assay and purification of recombinant proteins, prepare buffers and solutions the same day of use. Standard laboratory equipment is required including table top centrifuges, rotating mixers, digital heater block incubator, designated area for handling ^{32}P, equipment for cell culture and protein detection, gel dryer, X-Ray films and X-Ray film processors and chemicals.

2.1 Plasmids and Purified Proteins

1. Commercially purified recombinant human active JNK1 (Human JNK1, MEKK2-MKK7 activated, untagged, expressed in *E. coli*, full length, amino acids M1-Q384, as in NCBI/Protein entry NP_002741.1)
2. pET2b-PKM2 plasmid expressing (6x)-histidine-tagged human PKM2 cDNA [10].
3. pWPI-FLAG-tagged MKK7-JNK1α1 plasmid expressing human JNK1 constitutive active (JNK1^{CA}) cDNA [9,10].

2.2 Expression and purification of histidine-tagged recombinant proteins

1. Chemically competent *E. coli* BL21(DE3) cells: *F-ompT hsdSB (rB-mB-) gal dcm* (DE3) strain.
2. Lysogeny broth (LB): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl. LB needs to be autoclaved. Add fresh kanamycin (50 µg/mL final concentration) before use.

3. 1M $MgCl_2$
4. Commercially available SOC Medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose.
5. Commercially available LB agar plates containing 50 µg/mL kanamycin
6. 18 Gauge syringe needles and 10 mL syringes with Luer-lock tip.
7. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) (1M stock).
8. Bacteria Lysis Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 5 mM Imidazole, add fresh 0.1% 2-Mercaptoethanol
9. Wash Buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 20 mM Imidazole.
10. Elution Buffer: 50 mM NaH_2PO_4, 250 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 500 mM Imidazole.
11. Dialysis buffer: 10 mM HEPES pH7.5, 25 mM NaCl, 10 mM MgCl2.
12. Ni-NTA Spin Columns for rapid purification of 6xHis-tagged proteins from small-scale expression cultures.
13. Ultrasonic Cell Disruptors
14. Spectrophotometers and accessories for OD_{600} readings for bacteria cell growth density
15. 10 mg/mL Lysozyme stock solution

2.3 Ectopic expression and purification of JNK1 constitutive active ($JNK1^{CA}$) in HEK293T cells

1. Human embryonic kidney 293T (HEK293T) cell lines (Cellosaurus ID: CVCL_0063) [11].
2. NP-40 lysis buffer: 20 mM HEPES pH 8.0, 350 mM NaCl, 20 % Glycerol, 1% NP-40, 1 mM $MgCl_2$, 0.2 mM EGTA pH 8.0, add 1 tablet of EDTA-free protease inhibitor cocktail in 50 mL and store in aliquots -80°C until use. Prepare fresh the amount needed supplemented with: 1 mM DTT, 1 mM sodium orthovanadate, 50 mM sodium

fluoride, 10 μM chymostatin, 0.002 mg/mL leupeptin, 0.002 mg/mL antipain and 1 mM Phenylmethylsulfonyl fluoride (PMSF).

3. 1M Calcium chloride ($CaCl_2$)
4. 2x BES-buffered solution (BBS): 50 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 280 mM NaCl, 1.5 mM Na_2HPO4 pH 6.95. Dissolve the components in 800 mL ultrapure water first, then adjust the pH to 6.95-6.98 (pH is critical) at room temperature with 1 N NaOH, next adjust final volume (1 L). Sterile filter the solution (0.45 μm nitrocellulose filter) and store in aliquots at -20°C.
5. Tissue-culture treated culture 10 cm dishes
6. HEK293T cell culture medium: Dulbecco's modified Eagle's medium (DMEM, 25 mM glucose) supplemented with 10% Fetal Bovine Serum (FBS) and standard antibiotics.
7. 0.05% Trypsin-EDTA
8. Monoclonal ANTI-FLAG M2 antibody produced in mouse Affinity Agarose Gel
9. Immunoprecipitation (IP) M2 buffer: 20 mM Tris.Cl pH 7.5, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA pH 8.0, 0.5% NP-40, add 1 tablet of EDTA-free protease inhibitor cocktail in 50 mL and store in aliquots -80°C until use. Prepare fresh the amount needed supplemented with: 30 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 10 mM chymostatin, 0.002 mg/mL leupeptin, 0.002 mg/mL antipain, 1 mM PMSF.

2.4 Purification of endogenous active JNK1 in lysates of human cancer cells

1. Purified mouse anti-human active JNK1, clone G151-333 [11].
2. Matching IgG1 antibody isotype as control
3. Pre-washed protein A/G PLUS-Agarose
4. IP M2 buffer (see section 2.3.9).

2.5 PKM2 phosphorylation assay

1. [6x] Kinase Buffer (prepare fresh): for 5 mL final volume solution use 3125 µL ultrapure water, 600 µL 1M Hepes buffer, 600 µl 1M $MgCl_2$, 600 µL 1M β-glycerophosphate, 60 µL 1M DTT, 15 µL 100mM sodium orthovanadate.
2. 50 mM Hepes buffer
3. Deoxyadenosine triphosphate (dATP) (cold ATP)
4. ATP, [γ-^{32}P]- 3000Ci/mmol 5mCi/mL EasyTide Lead, 250 µCi (hot ATP)
5. ATP mix: 5 µL/each samples purified water, 0.3 µL/each samples 1mM dATP, 0.5 µL/each samples 5mCi/mL [γ-^{32}P]-ATP.
6. Capillary pipette tips for gel loading
7. 4x protein loading buffer: make a solution containing 5 mL 10% SDS, 2.5 mL 1M Tris-HCl pH 6.8, 2.5 mL Glycerol, 0.015 g brome phenol blue and store at room temperature until use. Store in the dark. Prepare fresh the amount needed supplemented with 20% 2-Mercaptoethanol by mixing on a rotating mixer.

2.6 SDS-PAGE, Coomassie blue staining and autoradiography

1. 10% Sodium Dodecyl Sulfate (SDS), electrophoresis grade
2. 5x Tris-glycine buffer: dissolve 15.1 g Trizma Base, 94 g Glycine and 50 mL 10% SDS in 1 L final volume of ultrapure water.
3. 10% Separating gel: In a 15 mL conical tube mix 4 mL ultrapure water, 3.3 mL of 30% acrylamide/bis-acrylamide solution, 2.5 mL of 1.5 M Tris–HCl, pH 8.8, 100 µL of 10% SDS, 100 µL of 10% ammonium persulfate (APS), and 15 µL of Tetramethylethylenediamine (TEMED).
4. 5% Stacking gel: In a 15 mL conical tube mix 2.7 mL ultrapure water, 670 µL 30% acrylamide/bis-acrylamide, 500 µL 0.5 M Tris-HCl pH 6.8, 500 µL 10% SDS, 40 µL of 10% SDS, 40 µL 10% APS and 10 µL TEMED.
5. Pre-stained molecular weight protein ladder.

6. Protein gel electrophoresis chamber systems including including combs, casting stand, glass plates, buffer tank.
7. Digital power supply.
8. Wash containers.
9. 25-100°C heat block
10. X-ray films and cassettes.
11. Gel dryer system
12. X-ray film processor
13. Coomassie blue: to prepare 1 L of a staining solution, dissolve 1 g of Coomassie R250 in 300 mL of methanol. Then add 650 mL of ultrapure water and 50 mL of acetic acid. Stir the solution on a magnetic stirrer for 2 hours.

3. Methods

Carry out all procedures wearing appropriate personal protective equipment (PPE) and following all your local guidelines for health and safety at work.

3.1 Expression of histidine-tagged recombinant proteins

1. Thaw competent BL21 cells on wet ice and gently mix the cells. Then make 50 µL aliquots in a pre-chilled 14mL round bottom polypropylene test tube with Snap Cap and keep on wet ice.
2. Add 1 µL of pET28b-PKM2 plasmid directly into competent cells. Mix well by gently flicking tube several times.
3. Incubate the cells on ice for 30 minutes.
4. Heat-shock the cells for exactly 30 seconds in a 42°C water bath.
5. Do not mix or shake the tube.
6. Incubate the cells on ice for 2 minutes.
7. Add 1 mL of room-temperature commercially SOC medium

8. Place the tube in a shaking incubator and set the shaker at 225 rpm for 1 hour at 37°C.
10. Spread 20 µL of transformed cells from each transformation reaction on separate LB agar plates containing kanamycin.
11. Invert the plates and incubate overnight at 37°C.
12. Add a tip of bacteria into a 200 mL of LB + kanamycin in a 1.5 L flask
13. Culture the bacteria overnight at 37°C in a 225-rpm shaking incubator.
14. Dilute 1:50 the growing bacteria in 50 mL of fresh LB broth supplemented with kanamycin and filtered-sterile 2 mM $MgCl_2$ (e.g. use 0.1 mL 1M stock $MgCl_2$ in a 50 mL LB final volume).
15. Growth bacteria at 37°C in a 225-rpm shaking incubator
16. From a growth culture, at the periodic intervals (20-30 minutes) take 1 mL aliquot and check the optical density of bacteria culture using a spectrophotometer for OD600 readings. Follow the vendor instruction to operate the machine
17. Growth bacteria until reach OD_{600} = 0.4.
18. Then, induce the bacteria with 0.5 mM IPTG (25 uL 1M IPTG in 50 mL culture volume) and growth for 6 hours at room temperature or overnight at 18°C.
19. Pellet bacteria in 250 mL conical tubes using a table top centrifuge at 4000 x g for 20 minutes; remove supernatant and snap-freeze the bacteria pellet in dry-ice (alternatively store -80°C, if necessary) (**Figure 1**).

3.2 Purification of histidine-tagged recombinant PKM2 proteins (His-PKM2)

1. Thaw and resuspend IPTG-induced bacteria pellet in Bacteria Lysis Buffer supplemented with lysozyme (0.1 volume of a 10 mg/mL lysozyme solution in 25 mM Tris-HCl, pH 8.0). For a pellet of bacteria obtained culturing 50 mL of BL21, resuspend pellet in 1.0 mL of Bacteria Lysis Buffer. Use a 10 mL syringe capped with a 18G needle to resuspend the bacteria.

2. Sonicate the cell suspension using an Ultrasonic Cell Disruptor (settings: total time 10min, pulse 50 seconds ON, 30 seconds OFF, amplification 40%). Operate in a cold room wearing appropriate PPE. Alternatively, while sonicating the solution adapt the conical tube on a bucket with wet ice to maintain the bacteria lysate at 4°C.
3. Clear the lysates by centrifuge at 12,000 x g for 20 min at 4°C.
4. Equilibrate a Ni-NTA spin column with 600 µL Bacteria Lysis Buffer
5. Centrifuge for 2 min at 890 x g.
6. Load up to 600 µL of the cleared lysate containing the 6xHis-tagged protein onto the pre-equilibrated Ni-NTA spin column. Centrifuge for 5 min at 270 x g.
7. Wash the Ni-NTA spin column twice with 600 µL of wash buffer.
8. Centrifuge for 2 min at 890 x g.
9. Elute the recombinant protein twice with 300 µL of Elution buffer.
10. Centrifuge for 2 minutes at 890 x g and collect the eluate. This is you're His-PKM2 recombinant protein. Add 10% of glycerol, mix well and store at -80°C for future use.

3.3 Ectopic expression of JNK1 constitutive active ($JNK1^{CA}$) in HEK293T cells

1. Propagate HEK293T cells in DMEM culture media.
2. Growth HEK293T cells until reach a 70-80% confluency, then passage cells by washing with PBS twice and detaching with 0.05% Trypsin/EDTA (see **Note 1**).
3. Plate 2 x 10^6 HEK293T cells on 10 cm tissue-culture treated dishes one day prior transfection.
4. Transfect HEK293T cells with 30 µg total DNA containing 15 µg pWPI-FLAG-$JNK1^{CA}$ and 15 µg pWPI-empty vector (see **Note 2**) (**Figure 1**)
5. Replace the transfection mixtures with fresh culture media after 12-16 hours of transfection.
6. Growth transfected cells for additional 48 hours post transfection.
7. Remove media and collect cells by scraping them in 50 mL conical tubes. Wash dishes with cold 1X PBS and collect any cells. There is no need to detach them by

trypsin, as this could interfere with the activation of JNK, a stress activated protein kinase.

8. Pellet cells in a 50 mL conical tube by centrifuge at 100-200 x g for 5-10 minutes.
9. Wash pellet in 800 µL ice-cold PBS and transfer cells into a 1.5 mL conical tube.
10. Proceed to purification of FLAG-JNK1^{CA} (see section 3.4).

3.4 Purification of ectopically expressed FLAG-JNK1^{CA}

1. Lysate the JNK1^{CA}-expressing HEK293T cells (see section 3.3) in NP-40 lysis buffer by adding 3x volumes of the corresponding amount of the pelleted cells.
2. Pipette up and down vigorously making sure all the cell pellet is in suspension.
3. Incubate on wet ice for 10 minutes
4. Snap-freeze in dry ice or freeze at -80°C (store if necessary).
5. Thaw the lysates and centrifuge for 15 minutes at 21,000 x g at 4°C.
6. Collect the supernatant in a clean 1.5 mL tube. This is your cleared cell extract.
7. Obtain protein concentration using conventional protocols by comparing the assay response of a sample to that of a protein standard whose concentration is known (i.e. Bradford Protein Assay) [12].
8. From the stock take the amount needed (5 µL x *n* samples) of anti-FLAG M2 affinity gel (resin) and wash with M2 buffer.
9. Centrifuge the resin for 30 seconds at 5,000-8,200 x g. add 1 mL of M2 buffer and aliquot equally amount in *n* test tubes.
10. Add 200-400 µg of cellular lysates
11. Incubate on a rotating mixer for 4 hours at 4°C.
12. Centrifuge the resin-containing FLAG-JNK1^{CA} for 30 seconds at 5,000 x g, aspirate the supernatant so that only a few mm of supernatant is left above the sedimented resin; add 1 mL of M2 buffer. Repeat two times this step.
13. Centrifuge, aspirate the supernatant and wash the resin with 1 mL of 50 mM Hepes buffer.

14. Pellet resin by centrifugation at 5,000 x g for 1 minute at 4° C.
15. Dry the sedimented resin using capillary pipette tips for gel loading. This is now ready to be used for the kinase activity.

3.5 Purification of endogenous active JNK1

1. Lysate cancer cells expressing active JNK protein in NP-40 lysis buffer by adding 3x volumes of the corresponding amount of the pelleted cells.
2. Pipette up and down vigorously making sure all the cell pellet is in suspension.
3. Incubate on wet ice for 10 minutes
4. Snap-freeze in dry ice or freeze at -80°C (store if necessary).
5. Thaw the lysates and centrifuge for 15 minutes at 21,000 x g at 4°C.
6. Collect the supernatant in a clean 1.5 mL tube. This is your cleared cell extract.
7. Obtain protein concentration as described in section 3.4.7
8. From the Protein A/G PLUS-Agarose stock collect the amount needed (20 µL x *n* samples) and wash with M2 buffer.
9. Centrifuge the resin for 1 minute at 1,000 x g.
10. Add 1 mL of M2 buffer and wash again. Repeat washing three times. After the last wash, aliquot equally amount in *n* test tubes.
11. Add 1 µL (i.e., 1 µg) purified mouse anti-human active JNK1, clone G151-333. If required, use an isotype matching antibody in control test tubes.
12. Incubate on a rotating mixer for 1 hours at 4°C to allow the binding of the antibody to the beads.
13. Add 600-800 µg of cellular lysates and incubate on a rotating mixer for 4 hours at 4°C.
14. Centrifuge the active-JNK1-coupled beads for 1 minute at 1,000 x g. Aspirate the supernatant so that only a few mm of supernatant is left above the sedimented resin; add 1 mL of M2 buffer. Repeat two times this step.

15. Centrifuge, aspirate the supernatant and wash the beads with 1 mL of 50 mM Hepes buffer.
16. Centrifuge again, aspirate and dry the beads using capillary pipette tips. This is now ready to be used for the kinase activity.

3.6 In vitro Kinase Assay to assess PKM2 phosphorylation by JNK

Kinase assay is used to detect the activity of a specific kinase (i.e. JNK) to phosphorylate a given substrate (i.e. PKM2). In this protocol, the active JNK kinase removes the radioactively labelled phosphate group from [γ-^{32}P]-ATP and adds it to the kinase target PKM2. Therefore, it is essential to perform the following steps in a designated Radioactive Materials (RAM) area, behind shielding and wearing appropriate PPE and personal chest and finger dosimeters (**Figure 1**).

1. For each test tube prepare a Kinase Reaction Mix containing 5 µL [6x] kinase buffer, 5 µL His-PKM2 [1 mg/mL], 5 µL ATP mix and 15 µL H_2O. Vortex and short spin before opening test tubes containing RAM.
2. Add 30 µL Kinase Reaction Mix to each tube containing M2 anti-FLAG resin-bound FLAG-JNK1^{CA} or immunoprecipitated active JNK1 or 0.1 µg of purified active-JNK1.
3. Incubate at 30°C for 20-40 minutes.
4. Every 10 minutes, gently tap the test tubes by hand to mix the beads/resin with the supernatant liquid.
5. Stop reactions by the addition of 5 µL of 4x protein loading buffer.
6. Denature samples by boiling at 95°C for 5 minutes (see **Note 3**).
7. Incubate on wet ice for 5 minutes. Short spin and load the supernatant in a 10% SDS-PAGE gel. Do not load the resin/beads (when applicable) (see **Note 4**) (**Figure 1**).

3.7 Electrophoresis, gel drying and autoradiography

1. Prepare a 10% SDS-PAGE gel, assemble the gel chamber, and add 1x running buffer using standard procedures [13].
2. Load equal amounts of the samples and 5 µL of pre-stained protein ladder on SDS-PAGE gel.
3. Adjust the power supply to obtain ~25 mA and run the electrophoresis until the dye front is 2-3 cm from the bottom of the gel.
4. After electrophoresis, open the gel casting and disassemble the two glasses.
5. Cut the bottom of the gel just above the dye front. This will remove the non-incorporated [γ-^{32}P]-ATP and will prevent the overexposure of the film at the bottom of the gel, which might interfere with the detection of the specific protein bands near the dye front.
6. Optional step: place the gel on a plastic box and stain with Coomassie blue for at least 2 hours on a gentle orbital shaking. De-stain the gel using the Coomassie blue solution without the R250 blue dye (**Figure 2**).
7. Adapt the gel on a piece of filter paper on one side and a piece of plastic wrap on the other side.
8. Dry the sandwich gel using a Vacuum Heated Gel Dryer, following the standard procedures for the given brand
9. Disassemble the dried gel from the gel dryer and place in an X-ray cassette along with an x-ray film to allow the radiation emitted to impress the film.
10. Place the cassette in -80°C for 20-40 minutes. The times it takes to impress the film will actually vary, as the freezing conditions enhance the impressed signal (**Figure 2**).
11. Develop film and analyse the image impressed onto the film
12. Discard the dried gel in RAM solid waste container.

4. Notes

1. To seed cells, use standard cell culture procedures keeping in mind that the number of cells seeded in a 10 cm plate is critical for the transfection efficiency and the yield of ectopic protein being produced. It is good practice to use HEK293T cells that are ~70-80% confluent to ensure a high yield. Gently aspirate cell media from culture flasks, ensuring to avoid cell detaching the flask. Wash flask with pre-warmth PBS, using the amount needed to cover your cell culture flask surface. Gently aspirate PBS and add pre-warmth trypsin/EDTA to the flask, using the amount needed to cover your cell culture flask surface. Gently rock your flask to cover the entire surface of the flask with trypsin/EDTA and place it into the incubator. After 2 minutes check the cells under the microscope to see if they have detached from the flask. This step is very critical: make sure that cells are disassembled from each other to make a single cell suspension. This is essential to obtain the wanted number of cells. Add 10 mL of cell media to your flask in order to neutralize trypsin/EDTA activity. Collect in a 50 mL conical tube. Take an aliquot to count the number of cells. Count and centrifuge the cells by following your routine procedures. Resuspend the cells using a 5 mL pipette, this will allow to obtain a single cells suspension. Resuspend the cell pellet with cell media to obtain a concentration of 2 x 10^6 cells/mL. Seed 1 mL for each plate using a 5 mL pipette (it is the most accurate of the large pipettes). Add 9 mL of cell media each plate and gently rock the plates to mix the cells with cell media. To obtain an even seeding of the cells rock the plates back and forth gently by making a cross. Avoid rocking in a circular orientation. After a thoroughly mixing, place the plates in the incubator.
2. This note is based on transfection of one 10 cm plate containing 2 x 10^6 cells HEK293T cells seeded the day before transfection. Scale up as necessary. Prepare plasmid DNA in H_2O (purified by endotoxin-free plasmid prep kit). Prepare a transfection mixture in a 14 mL round bottom polypropylene test tube containing: *a)*

30 µg total of plasmid DNA (15 µg pWPI-FLAG-MKK7-JNK1α1 + 15 µg empty vector); *b)* 375 µL cell culture purified water; *c)* 125 µL 1M $CaCl_2$; *d)* 500 µL 2x BBS. Add first water, then $CaCl_2$ and last BBS while holding tube on a vortex. Vortex strength need to be adjusted to avoid over spilling. Solutions have to be dropped into the solution directly, not onto the tube wall. Incubate at room temperature for 10 minutes. Add 1 mL of transfection mixture dropwise over the cell culture. Shake the plate or rock back and forth gently while dropping the solution directly in the centre of the plate. Incubate the transfected cells for 12-16 hours. The day after, change the medium and do not wash with PBS.

3. Flick tubes every 2 minutes. Make sure to use test tubes with hinged Safe-Lock lids when handlining samples with radioactive material. Pay extra caution when boiling tubes as they open easily.
4. The incubation in wet ice is an essential step to reduce the background noise. Do not forget to short spin to push down all the radioactive material.

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Figure legends

Figure 1. Simplified illustration of the protocol steps. 1) Obtain commercially available active JNK1 purified from baculovirus infected insect cell line (*Spodoptera frugiperda*, Sf9). 2) Alternatively, transfect DNA plasmid expressing FLAG-tagged JNK1 constitutive active (FLAG-JNK1^{CA}) into HEK293T cells and purify FLAG-JNK1^{CA} protein by immunoprecipitation using anti-FLAG affinity gel. 3) Alternatively, purify by immunoprecipitation endogenous active JNK1 from cellular lysates of cells known to express active JNK1. 4) Transfect DNA plasmid expressing 6x-His-tagged PKM2 into competent bacteria *E. Coli* cells (i.e BL21 strain) and induce protein expression by Isopropyl β- d-1-thiogalactopyranoside (IPTG) stimulation. Purify 6xHis-tagged PKM2 protein by using Ni-NTA spin columns. 5) Perform kinase assay by incubating 'active' kinase JNK1 with purified 6xHis-PKM2 as substrate in the presence of [^{32}P]-γ-ATP. 6) Load gels and separate proteins of interest. Next, dry gel and expose for radioactivity detection.

Figure 2. Active JNK1 phosphorylates PKM2 *in vitro*. *In-vitro* JNK1 Kinase Assay (K.A.) was performed by incubating increasing amount (μL) of recombinant activated JNK1 (rec. active JNK1 concentrated 1 μg/μL) with either purified 6xHis-PKM2 or GST-c-Jun as substrates in the presence of [^{32}P]-γ-ATP (see section 3.6). [^{32}P]-His-PKM2 denotes the phosphorylation of His-PKM2. [^{32}P]-GST-c-Jun denotes the phosphorylation of GST-c-Jun. Coomassie blue staining shows the purity and size of the recombinant proteins, His-PKM2 and GST-c-Jun. First lane shows protein ladder.

Figure 1

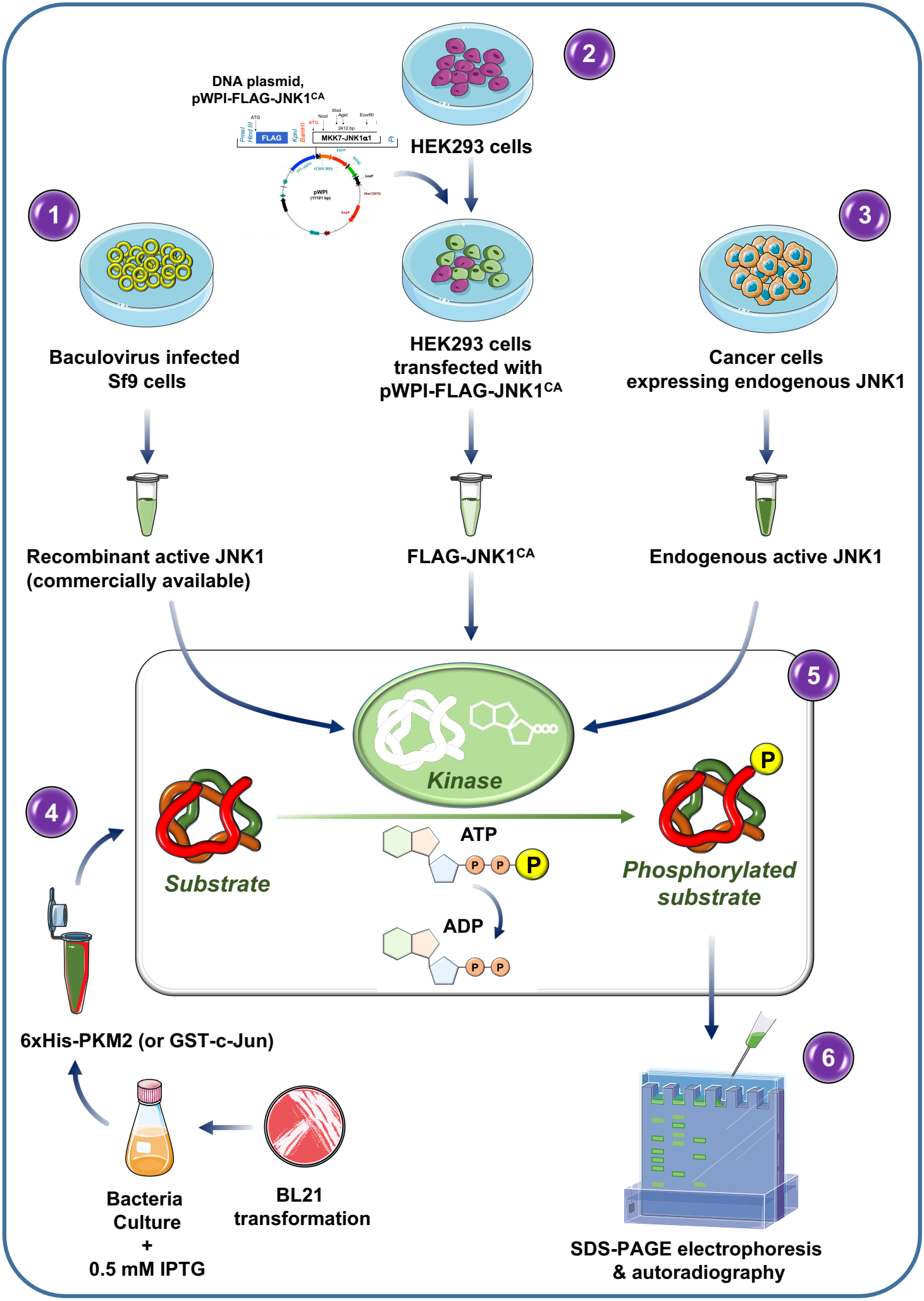
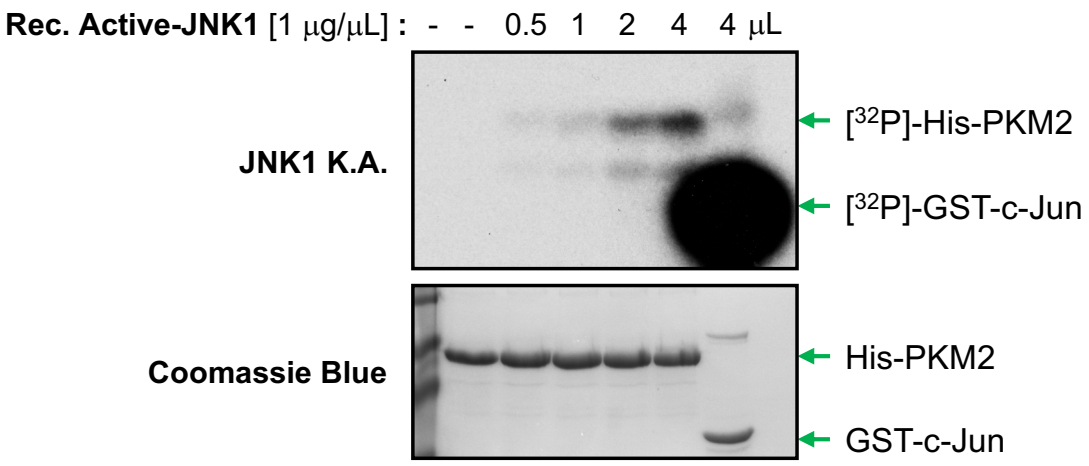

DNA plasmid,
pWPI-FLAG-JNK1CA
FLAG
MKK7-JNK1α1
HEK293 cells
1
2
3
Baculovirus infected
Sf9 cells
HEK293 cells
transfected with
pWPI-FLAG-JNK1CA
Cancer cells
expressing endogenous JNK1
Recombinant active JNK1
(commercially available)
FLAG-JNK1CA
Endogenous active JNK1
5
Kinase
Substrate
ATP
ADP
Phosphorylated
substrate
4
6xHis-PKM2 (or GST-c-Jun)
Bacteria
Culture
+
0.5 mM IPTG
BL21
transformation
SDS-PAGE electrophoresis
& autoradiography

Figure 2


Rec. Active-JNK1 [1 μg/μL] : - - 0.5 1 2 4 4 μL
JNK1 K.A.
[32P]-His-PKM2
[32P]-GST-c-Jun
Coomassie Blue
His-PKM2
GST-c-Jun