Chapter 30

Gel-Based Methods for the Investigation of Signal Transduction Pathways in *Trypanosoma brucei*

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Abstract

In the cell, reversible phosphorylation, controlled by protein phosphatases and protein kinases, initiates and regulates various signaling-dependent processes such as enzyme–substrate interactions, the cell cycle, differentiation, and immune responses. In addition to these processes, in unicellular parasites like *Trypanosoma brucei*, the causative agent of African sleeping sickness, additional signaling pathways have evolved to enable the survival of parasites in the changing environment of the vector and mammalian host. In this chapter, we describe two in vitro kinase assays and the use of the phosphoprotein chelator Phos-tag and show that these three polyacrylamide gel-based assays can be used for rapid target validation and detection of changes in phosphorylation.

Key words Signal transduction, Protein kinases, Protein phosphatases, Phospho substrate, Kinase assays, SDS-PAGE, Phos-tag

1 Introduction

Investigations of signal transduction pathways can be challenging in the trypanosome field due to the early divergence of these organisms from other eukaryotic lineages that may have resulted in the evolution of unique mechanisms and components. One of the main regulatory mechanisms of signaling events is the regulation of the phosphorylation states of the components involved. Two families of antagonistic enzymes are responsible for such regulation, that is, protein kinases that transfer the γ-phosphate of ATP molecules on the substrate acceptor residues and protein phosphatases that are responsible for the dephosphorylation of these proteins. Based on their analysis of the human phosphoproteome, in which 50,000 distinct phosphopeptides were detected, Sharma et al. [1] have revealed that at least 75% of the proteome is phosphorylated. In kinetoplastids, recent phosphoproteomic studies identified

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thousands of phosphosites [2–4], suggesting important roles for kinases and phosphatases regulating various signaling networks in these parasites.

Several high-throughput quantitative methods have been developed and are being used to study changes in phosphorylation (reviewed in [5]). However, there are cases when low cost, rapid, semiquantitative methods can be useful as the first step to identify a phosphosubstrate or the phosphorylation state of a target protein or to manually validate hits obtained by high-throughput technologies. Unfortunately, various phospho stains and commercial antibodies against phosphorylated S/T/Y residues of many signaling molecules cannot always be used on trypanosome cell lysates, for example, because of high background signal and low reproducibility.

Understanding the mode of regulation of kinases and phosphatases, as well as identifying their substrates, is therefore essential to unravel signaling pathways in trypanosomes. We here present a series of methods that can be easily implemented in any laboratory and used to determine the activity of protein kinases and phosphatases.

Kinases are often expressed at low level to facilitate the regulation of these important enzymes, rendering their purification from a physiologically relevant system, such as the parasite itself, potentially challenging. Commonly, bacterial systems are used to express proteins at a high level. However, the main weakness of these expression systems appears when researchers need an active enzyme because often *Escherichia coli* (and other prokaryote expression systems) cannot be used to produce active kinase, for example, due to the lack of eukaryotic posttranscriptional modifications. To overcome this limitation, we describe the use of an insect expression system to produce active trypanosome enzyme and, in combination with site-directed mutagenesis, to generate mutated versions of the kinase.

We also describe two in vitro kinase assays to identify a generic substrate that could be used for a structure/function analysis of different mutant versions of the kinase expressed. In the first instance, we recommend to use the "Cold" in vitro kinase assay with a modified ATP containing a substitution of the terminal oxygen of the γ-phosphate with a sulfur, based on the approach developed by Allen et al. [6]. This modified γ-phosphate is transferred to the substrate, and after an alkylation step, the modified substrate can be detected by a western blot probed with a commercially available antibody recognizing thiophosphate. If a stronger signal is required, we also describe an alternative approach that uses classical radioactive ATP, labeled on the γ-phosphate by the ^{32}P isotope. This method is also based on the migration of the phosphorylated substrates on SDS-PAGE and their transfer onto PVDF membrane. The activity for this "Hot" kinase assay is detected on

X-ray sensitive film and the excised bands corresponding to the substrates quantified by a scintillation counter. The advantages of these two kinase assays are that they are both based on the use of western blots that allow the detection of any other proteins of interest with corresponding antibodies, even when expressed at low level.

As a last gel-based method and as the first step to analyze changes in phosphorylation in *Trypanosoma brucei* cell lysates, we also describe Mn^{2+}-Phos-tag SDS-PAGE [7]. This method avoids the use of radioactive or chemical labels and is based on the Phos-tag molecule (1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex). This acts as a chelator in the presence of two divalent metal ions (preferably Mn^{2+} and Zn^{2+}) and binds specifically the phosphate monoester group regardless of the amino acid sequence context, such that it is equally potent in the detection of phosphorylation of Ser/Thr/Tyr. The other advantage of the method is that it uses conditions almost identical to standard SDS-PAGE conditions and can be combined with western blotting to investigate protein phosphorylation following electrophoresis. When Phos-tag (and Mn^{2+}) is added to the separating gel, it binds to the phosphoproteins and slows their migration with respect to unbound nonphosphorylated proteins, enabling separation of phosphorylated and nonphosphorylated molecules. Phosphoprotein isotypes can be also detected as multiple bands, with differential migration in the same lane, when Phos-tag SDS-PAGE is followed by western blotting and antibodies are used to detect the target protein.

2 Materials

2.1 PCR/ Mutagenesis

- M13 Forward primer (-40) (TGTAAAACGACGGCCAGT).
- M13 Reverse primer (CAGGAAACAGCTATGACC).
- Site-directed mutagenesis Forward and Reverse primers.
- Taq DNA polymerase kit, NEB.
- Phusion DNA polymerase kit, Invitrogen.
- Gene of interest cloned in the desired intermediate plasmid backbone.
- pFastBac™.
- 200 μL PCR tubes.
- dNTPs 10 mM.
- DMSO.
- DpnI restriction enzyme.
- Nucleases-free water.

- Agarose gel at desired percentage, containing the DNA specific dye of your choice (e.g., ethidium bromide, SYBR Safe).
- Appropriate DNA ladder.
- TAE 10× buffer (use at 1×): 400 mM Tris-base, 200 mM acetic acid, 10 mM EDTA, solution adjusted at pH 7.6.
- Thermocycler (e.g., TProfessional Basic gradient PCR Thermocycler).
- Power supply.

2.2 Bacterial Transformation/ Culture

- Your purified pFastBac™ construct (200 pg/μL in TE buffer: 10 mM Tris–HCl, pH 8, 1 mM EDTA).
- Positive expression control (i.e., pFastBac™1-Gus, used as a control for transposition).
- MAX Efficiency® E. coli DH10Bac™ chemically competent cells.
- *E. coli* XL1-Blue competent cells.
- LB agar plates containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL Bluo-gal, and 40 μg/mL IPTG to select for DH10Bac™ transformants.
- LB agar plates containing 50 μg/mL ampicillin.
- LB liquid medium (using same antibiotic concentration as for the plates when required).
- Super Optimal broth with Catabolite repression (SOC) Medium, Invitrogen.
- 42 °C water bath.
- 37 °C shaking and nonshaking incubator.

2.3 Insect Cell Culture/Infection/ Pulldown

- Sf9 cells.
- Sf-900 II serum-free medium (SFM) supplemented with 50 units/mL penicillin and 50 μg/mL streptomycin (final concentration).
- Grace's Medium.
- Cellfectin® Reagent.
- Purified bacmid.
- 0.2 μm filter (low protein binding).
- Fetal bovine serum.
- Radioimmunoprecipitation assay (RIPA) lysis buffer: 25 mM Tris–HCl, pH 7–8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, protease inhibitor cocktail.
- Dynabeads™ Protein G–coupled magnetic beads, Invitrogen.
- Purification magnet.

- Elution buffer: 100 μL 25 mM Tris–HCl, pH 7.4 and 10% glycerol.
- 15 and 50 mL Falcon tubes.
- Eppendorf tubes.
- Centrifuges (benchtop mini centrifuge for 1.5 mL tubes and centrifuge suitable for 15 and 50 mL tubes).
- Vortex.
- 200 and 500 mL glass bottles (Autoclaved).
- 6-well culture plates.
- 27 °C humidified incubator.
- 27 °C shaking incubator.

2.4 Kinase Assay

- MOPS kinase buffer 1×: 50 mM MOPS (3-(*N*-morpholino) propanesulfonic acid) adjusted at pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$.
- 50 mM PNBM (*p*-nitrobenzyl mesylate) in DMSO [6].
- 10 mM (ATP)-γ-S in distilled water (ddH_2O) [6].
- 250 μCi of 1 mCi [γ-^{32}P]-ATP (3000 Ci/mmol).
- Dephosphorylated MBP (myelin basic protein).
- Histone H1.
- Histone cores H2A, H2B, H3, H4.
- Dephosphorylated casein.
- ß-Casein.
- Recombinant *Mus musculus* caspase 9 (Casp9, first 200 amino acids).
- X-ray film developer.
- X-ray film.
- Scintillation counter.
- Scintillation vials.

2.5 Phos-Tag Assay

2.5.1 Sample Preparation

- *Trypanosoma brucei* cell lines: pleomorphic *Trypanosoma brucei* EATRO 1125 AnTat1.1 90:13 (TETR T7POL NEO HYG) [8] or double marker 29-13 procyclic form [9] trypanosomes, cultured in vitro in HMI-9 [10] medium at 37 °C under 5% CO_2 or in SDM-79 [11] medium at 27 °C, respectively.
- PBS (phosphate-buffered saline): 137 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.6.
- 50 mL Falcon tubes.
- Centrifuge capable of centrifuging 50 mL Falcon tubes at 1500 × *g*.

- Tabletop centrifuge capable of centrifuging 1.5 mL Eppendorf tubes and reaching 6200 × *g*.
- 1.5 mL Eppendorf tubes.
- Laemmli 6× sample buffer: 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, bromophenol blue, add β-mercaptoethanol (5%) immediately prior to use (50 μL in 1 mL).

2.6 SDS-PAGE and Western Blot

- Laemmli 6× sample buffer.
- Appropriate prestained and unstained protein ladders.

2.6.1 General

- ddH_2O.
- Transfer buffer 10×: 250 mM Tris–HCl, 1.92 M glycine (1×: 25 mM Tris–HCl, 192 mM glycine + 20% methanol).
- Methanol.
- Odyssey® Blocking buffer, Li-COR.
- TBS (Tris-buffered saline): 50 mM Tris–HCl, pH 7.5, 150 mM NaCl.
- Whatman 3MM Chr Chromatography Paper (GE Healthcare).
- Secondary antibodies conjugated to fluorescent dyes (i.e., secondary antibody: IRDye® 800CW Goat anti-Mouse IgG (H + L) (Li-COR Biosciences product n°: 926-32210, in 1:7000 dilution), anti-rabbit red IRDye® 680RD Goat anti-Mouse IgG (H + L) (Li-COR Biosciences product n°: 925-68070, in 1:7000 dilution)).
- Polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore).
- SYPRO Ruby stain.
- SYPRO fixing solution: 7% acetic acid, 50% methanol.
- SYPRO destaining solution: 7% acetic acid, 10% methanol.
- Ponceau S stain: 0.4% Ponceau S and 3% trichloroacetic acid.
- Microwave oven.
- Typhoon scanner, GE Healthcare (or any other fluorescent/UV gel imager system).
- Li-COR Odyssey® imager system (or any other fluorescent western blot imager system).
- Power supply.
- Protein gel electrophoresis chamber system.
- Mini Trans-Blot apparatus (Bio-Rad).
- Icepack.
- Rocking table.

2.6.2 Kinase Assays

- NuPAGE® Bis-Tris polyacrylamide gradient gels 4–12% (ThermoFischer Scientific).
- NuPAGE® MES 20× running buffer (ThermoFischer Scientific).
- TBS 0.1% Tween: TBS + 0.1% Tween.
- The primary antibodies αBB2 (1:5, [12]); αGST (1:1000); αThioP (1:1000, Anti-Thiophosphate ester clone 51-8, Abcam).

2.6.3 Phos-Tag Aassay

- 5 mM Phos-tag™ Acrylamide AAL-107 aqueous solution 0.3 mL manufactured by Wako Nard Institute and distributed by Alpha Laboratories.
- 10 mM $MnCl_2$ stock solution.
- Separating gel buffer: 1.5 M Tris–HCl, pH 8.8, 0.4% SDS.
- Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8, 0.4% SDS.
- 30% w/v acrylamide/bisacrylamide ratio 37.5:1.
- 10% ammonium persulfate (APS).
- *N*′,*N*,*N*,*N*′-Tetramethylethylene-diamine (TEMED).
- Bio-Rad PROTEAN II apparatus (glass plates, combs, casting stand, clamps).
- Running buffer 10× (use 1×): 1.92 M glycine 0.25 M Tris–HCl, pH 8.3.
- TBS 0.05% Tween: TBS + 0.05% Tween.
- Li-COR Block 50% Odyssey® block (Li-COR Biosciences) in TBS.

  Primary antibodies: anti-rabbit *Tb*PGKC (Tb927.1.700, 47 kDa, 1:75,000 dilution), anti-rabbit *Tb*PEX14 (Tb927.10.240, 39.9 kDa, 1:10,000 dilution), anti-rabbit *Tb*PIP39 (Tb927.9.6090, 39 kDa, 1:750 dilution), anti-mouse *Tb*TAT (Tb927.1.2340, 49.7 kDa, 1:5000 dilution).

  Pretransfer treatment

- Appropriate containers.
- ddH_2O.
- 2 mM EDTA.

3 Methods

3.1 Site-Directed Mutagenesis

- The principle of this method is to introduce point mutations inside the gene of interest. To do so, two complementary

primers are designed, integrating the point mutation in the middle of their sequences. For the design of those primers, we advise to use the QuickChange primer design tool from Agilent (https://www.agilent.com/store/primerDesignProgram.jsp), which is a user-friendly interface that gives good results.

– The PCR-based reaction could be done from the final plasmid but would require full sequencing to ensure that no other mutations have been introduced. Therefore, we would advise to perform the reaction on an intermediate plasmid before the final cloning as this would only require validation sequencing of the mutated gene of interest.

3.1.1 PCR and DpnI Digestion

1. The PCR-based reaction is performed under the following conditions (*see* **Note 1**):

5× HF buffer (GC rich)	4 µL
DNA (50 ng)	*x* µL
Primer Fwd (10 µM)	1 µL
Primer Rev (10 µM)	1 µL
dNTP (10 mM)	0.4 µL
DMSO (3% final)	0.6 µL
H_2O (up to 20 µL)	*y* µL
Phusion DNA polymerase	0.2 µL

98 °C	2 min	
98 °C 60 °C 72 °C	10 s 30 s 10 min	18 cycles
72 °C	10 min	

2. After the PCR reaction, add 20 units of DpnI to the 20 µL PCR reaction and incubate at 37 °C for 1 h to digest the parental (i.e., nonmutated) methylated DNA (*see* **Note 2**).

3.1.2 Bacterial Transformation

1. Gently thaw the *E. coli* XL1 Blue cells on ice. For each sample reaction to be transformed, aliquot 100 µL of the competent cells into an Eppendorf tube.
2. Swirl the contents of the tube gently. Incubate the cells on ice for 10 min, swirling gently every 2 min.
3. Transfer 5 µL of the DpnI-treated PCR reaction product from each sample reaction into separate aliquots of competent cells.

4. Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 min.
5. Heat-pulse the tubes in a 42 °C water bath for 45 s.
6. Incubate the tubes on ice for 2 min.
7. Add 0.5 mL of LB broth or SOC medium to each tube, then incubate the tubes at 37 °C for at least 1 h, with shaking at 225–250 rpm.
8. Centrifuge the culture, discard the supernatant, resuspend the pellet in 100 μL of LB, and plate all of each transformation reaction on agar plates, containing the appropriate antibiotic for selection of the plasmid vector.
9. Leave the plates in a 37 °C incubator overnight.
10. On the next morning, select colonies and grow them for at least 5 h in LB medium containing the appropriate drug selection.
11. Extract the plasmid DNA with the protocol of your choice.
12. The desired mutation and the integrity of the rest of the gene should be assessed by sequencing.

3.2 SF9 Insect Cells Expression System

– The expression of protein kinases and phosphatases in insect cells requires several steps, including the generation of the bacmid that will allow the infection of the insect cells. After the first step of infection has been performed, the virus needs to be amplified to obtain a sufficient titer for the subsequent infection, necessary for protein expression. The last step of the process consists of cell lysis and the pulldown of the target protein using the method of choice. Here, we describe the purification of proteins tagged with the combination of a TY1-epitope tag and YFP tag, to allow rapid screening of infected cells and the production and effective purification of expressed protein using the anti-TY1 BB2 antibody and magnetic Protein G-coupled Dynabeads.

– A comprehensive and extended protocol, including troubleshooting, of the insect cell expression system can be found at: http://tools.thermofisher.com/content/sfs/manuals/bactobac_man.pdf. Therefore, we only briefly describe the different steps.

3.2.1 Transformation

1. Proceed to the transformation of the DH10Bac™ cells as described earlier, using 100 ng of the pFastBac™ constructs.

2. After the heat-shock and the addition of LB or SOC medium, allow the cells to grow for 4 h at 37 °C, with shaking at 225–250 rpm.
3. For each pFastBac™ transformation, prepare tenfold serial dilutions of the cells (10^{-1}, 10^{-2}, 10^{-3}) with LB or SOC medium. Plate 100 μL of each dilution on a LB agar plate containing the three selective antibiotics, Bluo-gal, and IPTG.
4. Incubate the plates for 48 h at 37 °C.
5. Pick ten white colonies and re-streak them on fresh LB agar plates containing the three selective antibiotics, Bluo-gal and IPTG. Incubate the plates overnight at 37 °C.
6. The colonies for which the white phenotype is confirmed are then inoculated in liquid culture containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, and 10 μg/mL tetracycline, followed by overnight culture at 37 °C.
7. Recombinant bacmid DNA is then isolated using the method of your choice, and the successful transposition of the gene of interest to the bacmid is assessed by PCR using the M13 Forward (-40) and M13 Reverse primers and Taq DNA polymerase, with the following conditions:

10× PCR Buffer (appropriate for enzyme)	2 μL
Recombinant bacmid DNA (100 ng)	*x* μL
Primer Fwd (Stock 10 μM)	1 μL
Primer Rev (Stock 10 μM)	1 μL
dNTP (10 mM)	0.4 μL
$MgCl_2$ (if required)	*y* μL
H_2O (up to 20 μL)	*z* μL
Taq DNA polymerase	0.2 μL

94 °C	3 min	
94 °C 55 °C 72 °C	45 s 45 s 5 min	25–35 cycles
72 °C	10 min	

8. Load 5–10 μL from the reaction mixture on an agarose gel, and if transposition has occurred, a band should be visible at ~2300 bp + the size of your insert (*see* **Note 3**). If the transposition failed, a band corresponding to the bacmid alone will be visible at ~300 bp.

3.2.2 Transfecting Insect Cells

1. In a 6-well culture plate, seed 9×10^5 Sf9 cells per well in 2 mL of growth medium containing antibiotics (e.g., Sf-900 II SFM containing 50 units/mL penicillin and 50 μg/mL streptomycin final concentration (*see* **Note 4**)). Allow the cells to attach at 27 °C for at least 1 h.
2. During this time, for each transfection, prepare the DNA:lipid complexes as follows:
   - Dilute 1 μg of purified bacmid DNA (do not exceed a volume of 10 μL) in 100 μL of unsupplemented Grace's Medium in an Eppendorf tube.
   - In a second Eppendorf tube, mix 6 μL of Cellfectin® Reagent with 100 μL of unsupplemented Grace's Medium.
   - Then, combine the two tubes (total volume is ~210 μL), mix gently, and incubate for 15–45 min at room temperature.
3. Wash the cells once with 2 mL of unsupplemented Grace's Medium and remove the wash medium.
4. Add 0.8 mL of unsupplemented Grace's Medium to each tube containing the DNA:lipid complexes, mix gently, and add the DNA:lipid complexes dropwise to each well containing cells.
5. Incubate in a 27 °C incubator for 5 h.
6. Remove the DNA:lipid complexes and add 2 mL of complete Sf-900 II SFM (containing antibiotics) to the cells.
7. Incubate in a 27 °C humidified incubator for 72 h or until signs of late viral infection are visible (*see* **Note 5**) (Fig. 1a).
8. Once signs of late stage infection (e.g., at 72 h post transfection) are observed, collect the medium containing virus from each well and centrifuge the tubes at $500 \times g$ for 5 min to remove cells and large cell debris.
9. Filter the supernatant through a 0.2 μm filter (low protein binding) and add 2% of fetal bovine serum, to protect virus from digestion by proteases.
10. Store this P1 viral stock at +4 °C, protected from light.
11. Optional: determine the titer of your viral stock by performing a Viral Plaque Assay. You should expect a viral titer around 10^6–10^7 plaque forming units (pfu)/mL.

3.2.3 Amplifying Baculoviral Stock

1. Prepare a 20 mL Sf9 cell suspension at 2×10^6 cells/mL (>97% viability (*see* **Note 6**)) in an autoclaved 200-mL glass bottle (*see* **Note 7**).
2. Add the appropriate volume of P1 viral stock to the suspension culture to obtain a multiplicity of infection (MOI) of 0.1 (*see* **Note 8**).

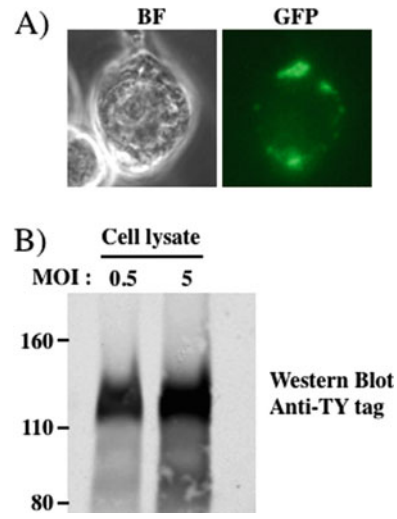

Fig. 1 Test of expression of a trypanosome protein kinase. (**a**) Infected insect cells can be quickly screened for TY1-YFP tagged target protein expression by mounting 10 μL of the insect cell culture, after 72 h of incubation, between a slide and coverslip and observing the GFP fluorescence under a fluorescence microscope. (**b**) Western blot of a crude insect cell lysate infected with a MOI of 0.5 or 5, where the TY1-YFP tagged target protein expression is revealed using an antibody raised against the TY1-tag

3. Incubate the cells for 48 h in a 27 °C incubator, with shaking at 120 rpm (*see* **Note 9**).
4. At 48–72 h post infection, collect the medium containing the virus from each bottle and transfer the medium to sterile Falcon tubes. Centrifuge the tubes at 500 × *g* for 5 min to remove cells and large debris.
5. Transfer the supernatant to fresh Falcon tubes. This is the P2 viral stock. Store at +4 °C, protected from light. For long-term storage, you may store an aliquot of the P2 stock at −80 °C, protected from light (*see* **Note 10**).
6. Optional: determine the titer of your P2 viral stock by performing a Viral Plaque Assay. You should expect a virus titer around 10^7–10^8 pfu/mL.

3.2.4 Expression and Purification of the Recombinant Protein

1. Prepare a 100 mL Sf9 cell suspension at 2.10^6 cells/mL (>97% viability) in an autoclaved 500 mL cylindrical glass bottle.
2. Add the P2 baculoviral stock to each well at the desired MOI (*see* **Note 11**) (Fig. 1b).
3. Incubate the cells in a 27 °C incubator, with shaking at 120 rpm (*see* **Note 9**).

4. Harvest the cells (for nonsecreted proteins) at the appropriate time (e.g., 24, 48, 72, 96 h post infection; this will need to be determined empirically, but a good starting point will be between 48 and 72 h).
5. Centrifuge the cells for 10 min at 500 × *g*.
6. Remove the supernatant and rinse the cells once with serum-free medium.
7. Lyse the cells with 15 mL of RIPA lysis buffer. Incubate on ice for 30 min.
8. Sonicate the samples to shear the DNA with the sonicator five times for 10 s ON followed by 20 s OFF at 5 Amp.
9. Clear the lysate by centrifugation for 15 min at 15,000 × *g* and 4 °C.
10. Keep the supernatant and discard the pellet (*see* **Note 12**).
11. For the pulldown of TY1-tagged protein, add the appropriate amount of αBB2 antibody (depending on the titer of the antibody) (*see* **Note 13**).
12. Incubate on a rotating wheel for 2 h at 4 °C.
13. Add 70 µL Protein G Dynabeads preequilibrated in lysis buffer.
14. Incubate for 1 h at 4 °C on a rotating wheel.
15. Pull down using a magnetic field and wash three times with 500 µL lysis buffer (for each wash, incubate on ice for 2 min).
16. Wash once with 500 µL of Elution buffer.
17. Native elution can be performed, with the beads, outside the magnetic field with 100 µL of Elution buffer.
18. Aliquot samples and freeze at −80 °C.
19. The pulldown quality is assessed by migration of the samples after SDS-PAGE:
    - Boil samples for 5 min in the presence of 1× Laemmli buffer.
    - Separate the proteins by SDS-PAGE.
    - Reveal the resolved proteins using SYPRO Ruby staining according to manufacturer's instructions. The relative quantity between different mutants can be assessed by this method, permitting determination of the correct dilutions for the future enzymatic assays (Fig. 2a).
    - Alternatively, proteins can be transferred onto PVDF membrane and the proteins detected by western blotting using appropriate antibodies (Fig. 2b).

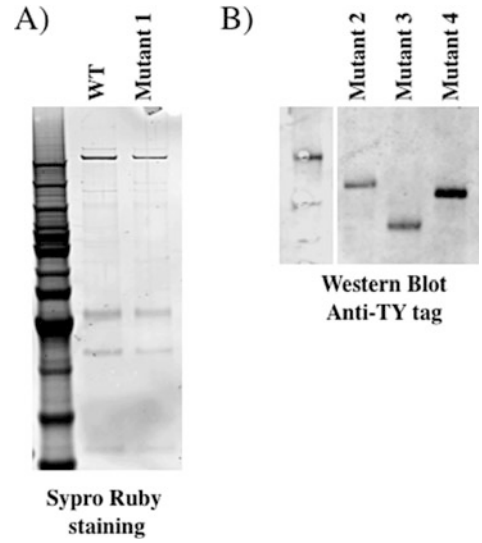

Fig. 2 Analysis of the quality of the pulldown from insect cells of the tagged protein kinase shown in Fig. 1, either as a wild-type or mutated protein. (**a**) Gel stained by SYPRO Ruby revealing the high quality of the purification of the wild-type and mutant 1 protein. (**b**) Alternatively, protein purification can be revealed by western blot here using an antibody detecting the TY1-tag incorporated into each expressed protein

3.3 Kinase Assays

3.3.1 "Cold" Kinase Assay

1. Prepare the "kinase mix" with the appropriate amount of MOPS kinase buffer 1× (*see* Subheading 2.4) and 5% of the purified kinase (including magnetic beads).
2. In a separate tube, prepare the "substrate mix" with 250 μM of (ATP)-γ-S, 200 μM ATP and the appropriate amount of substrate (*see* **Note 14**).
3. Start the kinase assay with mixing both the "kinase mix" and the "substrate mix" (*see* **Note 15**) and incubate for 30 min at 37 °C (*see* **Note 16**).
4. Stop the phosphotransferase reaction by incubating for 10 min at 95 °C.
5. Incubate the reaction with 5 mM (final concentration) PNBM for 2 h at 20 °C to initiate the alkylation reaction as described in Allen et al. [6].
6. The reaction is then stopped with 5 μL of Laemmli 6× loading buffer.

3.3.2 "Hot" Kinase Assay

If a more sensitive assay is required, we recommend to use the "hot" kinase assay, based on the presence of radioactive ATP, with the gamma phosphate group having a ^{32}P isotope.

1. As previously described, prepare the "kinase mix" with the appropriate amount of MOPS kinase buffer 1× (*see* Subheading 2.4) and 5% of the purified kinase (including magnetic beads).
2. In a separate tube, prepare the "substrate mix" with 1 mCi [γ-^{32}P]-ATP (3000 Ci/mmol), 200 μM ATP, and the appropriate amount of substrate.
3. Start the kinase assay with mixing both the "kinase mix" and the "substrate mix" (*see* **Note 15**) and incubate 30 min at 37 °C (*see* **Note 16**).
4. Stop the reaction with 5 μL of Laemmli 6× loading buffer.

3.3.3 SDS-PAGE and Transfer Onto PVDF Membrane

1. Boil the protein samples for 5 min in 1× Laemmli loading buffer.
2. Load the samples on a NuPAGE® gel 4–12% Bis-Tris and separate them by SDS-PAGE for 10 min at 100 V, followed by ~90 min at 120 V or until the desired protein separation is achieved.
3. Transfer the proteins onto PVDF membranes by the method of choice. We obtain good and reproducible results using wet transfer in 1× transfer buffer +20% methanol, for 2 h at 100 V (*see* **Note 17**).
4. Assess the quality of the separation and transfer by Ponceau S staining. Incubate the PVDF membrane for 5 min with 15 mL of Ponceau S, wash several times with ddH_2O or until the background staining remains stable and the protein bands become visible.
5. Images of the membrane can be captured at this point.

3.3.4 Phosphotransferase Activity Measurement

"Hot" kinase assay

1. ^{32}P incorporation is monitored by exposing an X-ray sensitive film to the membrane (*see* **Note 18**) at −80 °C.
2. After exposure, the bands corresponding to the substrates can be excised from the PVDF membrane and placed into individual scintillation vials.
3. Cherenkov radiation (as count per minute (cpm)) is quantified by a scintillation counter using the ^{32}P program, with three readings being taken for each sample (*see* **Note 19**).
4. If protein loading assessments require more sensitive methods than simple Ponceau S staining, a western blot analysis can be performed as described below for the "cold" kinase assay using appropriate antibodies, before excision of the bands from the membrane (Fig. 3).

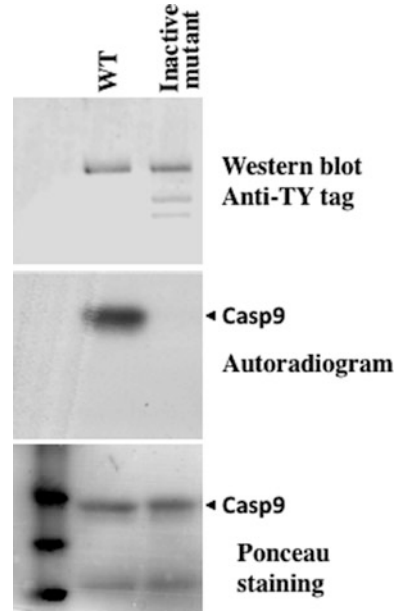

Fig. 3 "Hot" kinase assay of wild-type and an inactive mutant of the kinase expressed in Fig. 1, purified from insect cells and reacted against the Mus musculus Casp9 as a generic substrate. The loading of the kinase was revealed by western blot using the anti-TY1-tag antibody. The loading of the Casp9 was revealed by Ponceau S staining and the phosphotransferase activity of the kinase revealed by 5 h exposure of *X*-ray sensitive film (autoradiogram)

"Cold" kinase assay

1. Following Ponceau S staining, the membrane is blocked for 45 min at room temperature with Li-COR Odyssey® Blocking buffer (*see* **Note 20**).
2. Incubate the membrane with primary antibodies for 1–3 h at room temperature or overnight at 4 °C with agitation in 50% TBS-T and 50% Odyssey® Blocking buffer (*see* **Note 20**).
3. Use the following antibody dilutions: αBB2 (1:5, detecting the TY1-tag); αGST (1:1000); αThioP (1:1000, for the detection of the transferred thiophosphate).
4. Wash three times for 10 min with TBS-T at room temperature.
5. Incubate the membrane with secondary antibodies conjugated to a fluorescent dye diluted 1:5000 in 50% Li-COR Odyssey® Blocking buffer (*see* **Note 20**) and 50% TBS-T for 1 h at room temperature.
6. Wash three times for 10 min with TBS-T at room temperature.
7. Scan the membrane with a Li-COR Odyssey® imager system (*see* **Note 21**).

3.4 Phos-Tag Assay of Phosphorylated Proteins

3.4.1 Sample Prep

1. Harvest 2.5 × 10^7 cells in a 50 mL Falcon tube for 10 min at 1500 × *g*.
2. Pour off most of the supernatant from the cell pellet and resuspend in 500 μL of PBS.
3. Transfer all the resuspended cells to a labeled Eppendorf tube and spin down at 6200 × *g* for 5 min in a benchtop centrifuge.
4. Remove all the supernatant carefully and add 62.5 μL Laemmli 6× buffer and pipette up and down several times until the sample is less viscous.
5. Boil the sample for 5 min in a heating block, and either load the samples on SDS-PAGE gels or freeze them and store at −20 °C.
6. Load 2–4 × 10^6 of cells in Laemmli 6× buffer per lane for SDS-PAGE.

3.4.2 SDS PAGE Using Mini Protean Gel Equipment (Bio-Rad)

Gel preparation

1. Wash and clean the glass plates (with ddH_2O and 70% ethanol) and assemble the glass plate sandwich (*see* **Note 22**).
2. Mix together the solutions (*except TEMED*) required for the separating (Table 1) and stacking (Table 2) gels in sterile tubes. For a single 0.75-mm thick protein gel, prepare 4 mL separating solution and 1.5 mL stacking solution. Supplement the separating gel mix with 10 mM $MnCl_2$ and the Phos-tag gel mix with 10 mM $MnCl_2$ and 5 mM Phos-tag solutions (Fig. 4, Table 1) (*see* **Notes 23–25**).
3. Add TEMED to the separating solution, swirl the mixture and pipette the solution between the gap between the plates, leaving sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Retain the leftover stacking gel mix in the tube, to monitor polymerization.
4. Prior to polymerization, overlay the gel carefully with a layer of 70% ethanol (this prevents oxygen from inhibiting polymerization and also creates an even surface).
5. After polymerization is complete (this depending on the volume of APS and TEMED added), pour off the 70% ethanol.
6. Add TEMED to the stacking solution. Pipette the stacking gel solution directly onto the surface of the polymerized resolving gel, to reach the edge of the glass sandwich. Keep the remainder of the stacking gel mix in the tube, to monitor polymerization.
7. Insert a clean comb carefully to avoid trapping air bubbles (first lower one side, then the other into the stacking gel solution). Leave the gel to set at room temperature.

Table 1
Solutions used for a 10% 0.75-mm thick normal and Phos-tag (supplemented with 10 μM Phos-tag compound) resolving gels

	Normal gel	Phos-tag gel
H_2O	2.06 mL	2.05 mL
PhosTag reagent (5 mM)	0	10 μL
$MnCl_2$ (10 mM)	20 μL	20 μL
Lower gel buffer	1.25 mL	1.25 mL
Acrylamide	1.67 mL	1.67 mL
10% APS	33 μL	33 μL
TEMED	5 μL	5 μL

Table 2
Solutions used for one 0.75 mm stacking gel

	1 gel
H_2O	1.5 mL
Lower gel buffer	0.375 mL
Acrylamide	0.625 mL
10% APS	12.5 μL
TEMED	4 μL

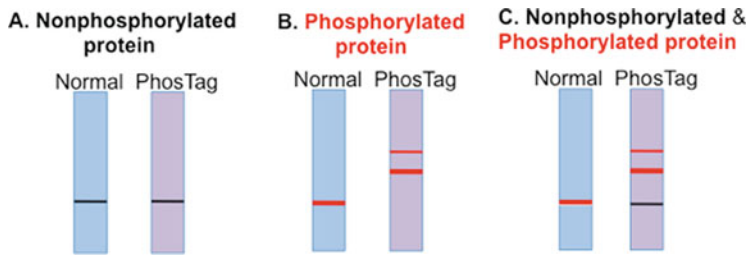

Fig. 4 (**a**) Nonphosphorylated "Protein X" (black band on schematic figure) migrates as a single band both on normal and PhosTag gel. (**b**) On a normal SDS gel, distinct phosphoprotein isotypes with different positions or numbers of phosphorylation migrate as a single band, but on PhosTag gel, they can also be separated depending upon the degree of phosphorylation. The thick red band denotes "Protein X" phosphorylated on a single residue, while the thin red band corresponds to a diphosphorylated "Protein X". (**c**) The mix of nonphosphorylated "Protein X" and phosphoprotein isotypes with different positions or numbers of phosphorylation migrate as a single band, but on a PhosTag gel, they can also be separated depending upon the degree of phosphorylation. Fastest migration: nonphosphorylated (black band), followed by uniphosphorylated (thick red band) and diphosphorylated "Protein X"

8. After the gel has polymerized, remove the comb and wash the wells with ddH_2O to remove any nonpolymerized acrylamide solution. The assembled gel sandwiches can be stored flat, wrapped in wet tissue in the fridge for few days.

Assembling, loading, and running the gel

1. Assemble the gel sandwiches in the electrophoresis apparatus (two gels can be run per tank; if only one gel is being run, a plastic buffer dam should be used to separate the upper and lower buffer in the tank).
2. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs.
3. Load up to 20 μL of sample to the wells of a 10-well gel (*see* **Note 26**).
4. Load 5–7.5 μL of appropriate protein marker to one lane of the gel (*see* **Note 27**). This allows estimation of resolved protein sizes and can be used to monitor the efficacy of transfer from the gel to the membrane after visualization by staining with Ponceau S stain. The use of prestained protein markers is not recommended with on Phos-tag gels, however these can be used on "normal" gels used as controls.
5. Run the gel at 100 V for 1.5–2.5 h or until the blue dye from the sample buffer reaches the very bottom of the gel (*see* **Note 28**).
6. Remove the glass plates from the electrophoresis apparatus and carefully take apart the glass–gel sandwich. Never use metal tools in this process; if needed, use the plastic wedge included with the Mini Protean II system.

Gel treatment before western blotting

1. After SDS-PAGE separation, rinse the gel in ddH_2O for 10 min, on a rocking platform, at room temperature.
2. Discard the ddH_2O and soak the gel in 2 mM EDTA for at least 20 min on a rocking platform at room temperature to chelate free Mn^{2+}. Omitting this step will reduce the efficiency of the protein transfer.
3. Discard the 2 mM EDTA solution and wash the gel with transfer buffer for 60 min on a rocking platform, at room temperature.
4. After this, the gel is ready for transfer.

Western blot

1. Cut four Whatman filter papers and a PVDF membrane into 8.5 × 5 cm pieces for a Bio-Rad minigel (use tweezers to handle these components). Use pencil to mark the membranes (*see* **Note 29**).

2. PVDF membranes should be activated for 60 s by submerging them in methanol. Soak the four Whatman papers, a PVDF membrane, and two sponges in transfer buffer before use. Always keep the PVDF membrane and Whatman papers wet throughout the process.
3. Assemble the gel sandwich for transfer in the order as follows (*see* **Note 30**):
   - The gel sandwich is prepared by placing the provided cassette in a small tray containing blotting buffer with the black side facing down. On top of the black side of the cassette, the other components were assembled in the following order: one of the fiber pads (provided with the Mini Trans-Blot apparatus (Bio-Rad)), two pieces of filter paper, the gel, the membrane, the other two pieces of filter paper, and the other fiber pad. To make sure that no bubbles are trapped between the different layers a 10-mL pipette is rolled on top of the assembled gel sandwich applying gentle pressure. The cassette was closed and placed in the apparatus, with the black side facing the black side (negative side) of the blotting device.
4. Fill the transfer tank with transfer buffer for 1 h at 80 V or overnight at 15 V. Avoid the buffer warming up during transfer using icepacks in the transfer tank and by placing the tank apparatus into wet ice in a large container.
5. After the transfer, disassemble the apparatus. Remove the PVDF membrane from the sandwich and place it in a small plastic tray.
6. Mark the PVDF membrane with pencil and stain the western blot with Ponceau S (to assist staining, place on a platform shaker for 5 min) to ensure the efficacy of transfer and to demonstrate even sample loading. Wash out the unspecific Ponceau staining with ddH_2O and take an image of the stained blot stained.
7. Wash off the Ponceau S solution with ddH_2O (by placing the blot on a platform shaker for 5 min and then by washing 3×).
8. Add 50% Li-COR block solution to the membrane in 1× TBS (note: different blocking solutions can be used depending the antibody used). Cover the tray and incubate the membrane with the blocking solution at room temperature for at least 45 min (or overnight in the cold room) on an orbital shaker.

Detection

1. Pour off the blocking solution (there is no need to wash if the primary antibody is diluted in the same blocking solution). Add primary antibody diluted in the blocking solution (*see* **Note 31**).

2. Incubate for a minimum of 1 h at room temperature (or overnight in the cold room) on an orbital shaker.
3. Wash the membrane 3× with TBS Tween (0.05% Tween)—each time add enough solution to cover the membrane, place it on a platform shaker for 10 min, then pour off the wash solution.
4. Add secondary antibody diluted in blocking solution.
5. Incubate for a minimum of 1 h at room temperature (or overnight in the cold room) on an orbital shaker.
6. Wash the membrane 3× with TBS Tween—each time add enough solution to cover the membrane, place it on a platform shaker for 10 min, pour off the solution.
7. The membrane is now ready to be scanned on the Li-COR/Odysseys® scanner.
8. The membrane can be washed in TBS Tween and dried by touching a paper towel for storage in cling film at −20 °C.

4 Notes

1. It is recommended to use a proofreading polymerase, such as Phusion DNA polymerase, to avoid mutations occurring at undesired position in the gene and/or the backbone plasmid.
2. Before bacterial transformation, the PCR reaction should be analyzed on an agarose gel by loading 5 μL of the reaction. A band at the expected size of the plasmid should be observed. If no band is observed, we would advise to repeat the PCR reaction with modified conditions. If a band is observed, proceed to the transformation of XL1 Blue bacteria.
3. The sizes indicated are the ones observed if you choose to use the pFastBac™1 plasmid. For other plasmids, you should refer to the manual with the link provided earlier.
4. We recommend the use of SF900 II for the culture of the insect SF9 cells, as this does not contain serum (SF9 cells have been adapted to culture without serum). This facilitates the subsequent steps of infection and protein purification. We also recommend to use this medium supplemented with 50 units/mL penicillin and 50 μg/mL streptomycin (final concentration) to avoid contamination.
5. The early infection stage is represented by growth arrest, followed by an increase in the cell and nucleus diameter, a granular appearance and a detachment of the cells from the plate. The very late stage of infection is characterized by cell lysis and a disappearance of the cell monolayer.

6. To assess the viability of the cells, dilute 5 μL of Trypan blue (stock 4%, final concentration 0.4%) with 45 μL of the cell culture. Pipette 10 μL of the mixture on a hemocytometer and count the cells under a microscope. The white/transparent cells are healthy cells, and the blue/dark cells are damaged or dead cells.
7. In our lab, we have found that the use of cylindrical glass bottles is most convenient for the culture of insect cells as they are inexpensive, cap sealed, and reusable and can be sterilized by autoclave. However, it is essential to thoroughly clean and rinse the bottles before autoclaving, in order to remove any trace of detergent, which is lethal for the insect cells.
8. To calculate the inoculum required, you can use the following formula:

$$\text{Volume required (mL)} = (\text{MOI (pfu/cell)} \times \text{number of cells})/\text{titre of viral stock (pfu/mL)}.$$

9. If you are using glass bottles as recommended, remember to slightly loosen the cap to allow gas exchange.
10. As previously mentioned, the viral stock can be sterilized with a low binding 0.2 μm filter, which may also increase the pfu/mL.
11. Different MOI can be tested, for example, 1, 2, 5, 10, 20, and the most suitable will need to be determined empirically for the protein of interest. However, a good starting point would be a MOI of 2.
12. Optional: protein samples can be resolved by SDS-PAGE and transferred onto PVDF membrane to analyze the protein expression using appropriate antibodies by western blot (pellets/flow throughs/washes can also be analyzed to see whether the protein is lost in the insoluble fraction or during the different purification steps).
13. If you choose to use other tags, conditions will have to be empirically tested with the appropriate antibody or methodology.
14. If the substrates of the kinase of interest are unknown, identifying a generic substrate will be essential and a good starting point would be to test the following molecules:
    (a) 10 μg dephosphorylated MBP.
    (b) 40 μg histone H1.
    (c) 1 μg histone cores H2A, H2B, H3, H4.
    (d) 20 μg dephosphorylated casein.
    (e) 1 μg ß-casein.
    (f) 1 μg of the recombinant Mus musculus caspase 9 (Casp9, first 200 amino acids).

15. Make sure that the final volume does not exceed 25 μL, to be able to load the entire reaction into the gel.
16. The optimal temperature and time should be adjusted for each individual protein kinase.
17. During the transfer procedure, it is important to maintain the temperature of the transfer buffer below 15 °C to avoid changes in the pH of the solution. To do so, simply place your transfer tank in an ice bucket during the transfer.
18. We highly recommend sealing the membrane in a transparent plastic envelope, before exposure to the X-ray films at −80 °C. This prevents any liquid contacting the film, which can create background signal.
19. When measuring the ^{32}P isotope by its Cherenkov radiation, it is not necessary to add any scintillation liquid into the vial before measurement to obtain a reliable and reproducible signal.
20. Other blocking buffers could be used but will require appropriate optimization before preforming the kinase assay.
21. Other fluorescent gel imagers could be used.
22. In our assays, to study the phosphorylation state of target proteins, we mixed the Phos-tag reagent in Bis-Tris acrylamide gels at an appropriate concentration, instead of buying the premixed Phos-tag precast acrylamide gels from the provider. This allowed us to screen various Phos-tag compound concentrations and various % acrylamide gels to identify the best resolution/separation of the target proteins.
23. A nonsupplemented "normal" gel (no Phos-tag reagent added) with identical % of acrylamide to the Phos-tag gel must be run in parallel in the same electrophoresis apparatus. The "normal" gel will be used as a control for the unaltered mobility of your target protein.
24. We have used various concentrations of Phos-tag (30, 40, 60, and 80 μM) and achieved various results (Fig. 5), depending on the target protein. According to our experiments, an increased concentration of Phos-tag reagent in the separating gel can provide better resolution and resolve two bands, which might appear as a single band at lower concentration (*see* Fig. 5: PEX14, 40 μM vs. 30 μM). We would recommend <40 μM Phos-tag as a starting concentration and, depending on the outcome, titrate the compound further to increase resolution.
25. High Phos-tag concentrations can negatively affect SDS-PAGE resolution and lead to distorted bands.
26. A recombinant version of the target protein produced in a prokaryote expression system should be loaded on the Phos-

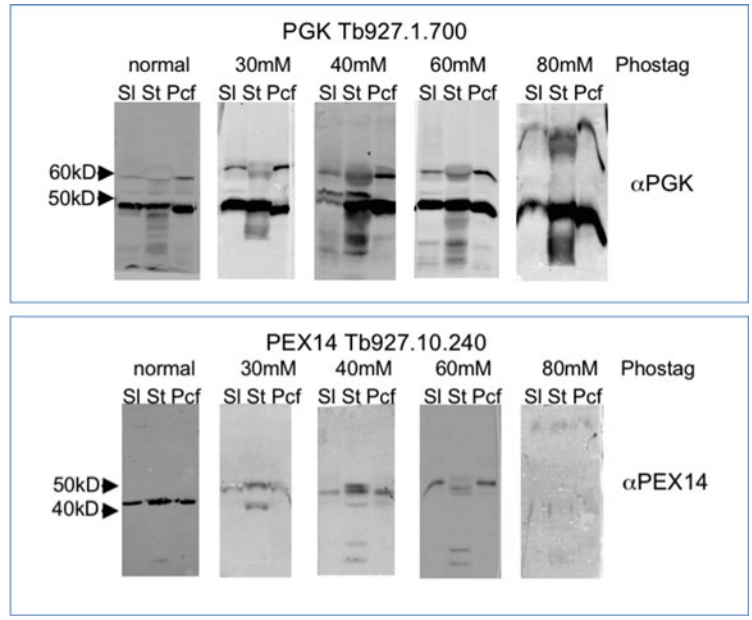

Fig. 5 Effect of increasing Phos-tag concentration on the migration of phosphoproteins. We used the Phos-tag SDS-PAGE method followed by western blotting to investigate how the phosphorylation of TbPGK and TbPEX14 changes in three different lifeforms (slender (sl), stumpy (st), and procyclic (pcf) forms) of *Trypanosoma brucei*. Trypanosome lysates equivalent to 2×10^6 cells were separated on 8% polyacrylamide gels and the Phos-tag gels were supplemented with 30–40–60–80 μM Phos-tag. No changes were detected in the TbPGK band pattern, but in the case of TbPEX14, the middle lane (st) and, possibly, the last lane (pcf) have more bands detected compared with the "normal" gel's band pattern. This suggests TbPEX14 is likely di or triphosphorylated in "st" and possible monophosphorylated in "pcf" The same protein is nonphosphorylated in "sl." The increasing concentration of Phos-tag has improved the separation of the target phosphoproteins (60 μM vs. 40 μM vs. 30 μM), but the highest concentration (80 μM) led to distorted bands

tag gel (and the "normal" gel, also) providing a negative control, revealing the migration of a nonphosphorylated version of the target protein.

27. Protein markers should be avoided (especially prestained markers, which can distort protein bands) because the protein separation on Phos-tag SDS-PAGE does not depend solely on molecular weight.
28. In our experiments, we found that for the best resolution, the gels need to be run very slowly at low voltage (100 V or lower).
29. After the SDS-PAGE separation, we used western blots probed with the appropriate antibodies for the detection of target proteins with altered mobility (phosphorylation).

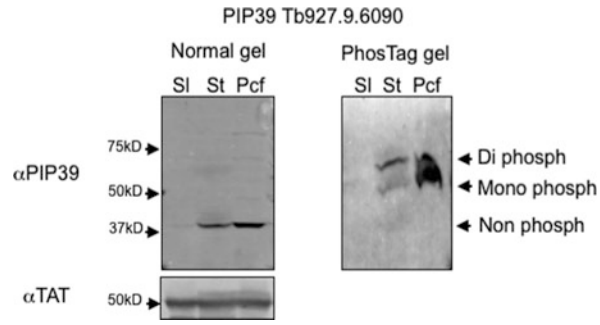

Fig. 6 TbPIP39 is differentially phosphorylated in slender, stumpy, and procyclic forms (*see* **Note 32**). *Trypanosoma brucei* lysates equivalent to 2×10^6 cells were separated on 8% polyacrylamide gels. The Phos-tag gel was supplemented with 40 μM Phos-tag compound

30. Wet overnight transfer provided better transfer of proteins from Phos-tag gels than the use of semidry apparatus.
31. For the success of this reasonably rapid and cost-effective method, it is important to have good antibodies. If these are not available, antibodies against tagged proteins can be used.
32. We used 8% 40 μM Phos-tag gel to investigate *Tb*PIP39 differential phosphorylation in slender, stumpy, and procyclic forms. According to the western blot probed with anti-*Tb*PIP39 antibody, we suggest that *Tb*PIP39 is phosphorylated on at least two residues in stumpy and procyclic forms. Analyzing the band pattern, we propose that the ratio of the phosphorylated residues differs in these two lifeforms (Fig. 6).

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