Preface

The wealth of primary information provided by genome sequencing projects in various species is of enormous potential value in our efforts to understand biological funtions and molecular interactions not only in normal development and cellular physiology, but also in diseases. However, utilization of these resources can come only from the development and application of a fully integrated set of molecular, biochemical, biophysical, and genetic skill bases. As key components of many cell signalling pathways, protein kinases are implicated in a broad variety of diseases, including cancers and neurodegenerative conditions, and offer considerable potential as tractable targets for therapeutic intervention. With these issues in mind, the present vlume has been compiled to provide examples of core skills required for analysis of knase-mediated signalling cascades, with particular emphasis on identification of proteins according to interactive relationships and analysis of functional properties of signalling proteins.

Compilation of Protein Kinase Protocols has been possible only as a result of the effort of all the contributors, and I am grateful to them for taking the time and having the patience to desseminate the detailied information required in order that others can succeed in the application of these techniques. Most importantly, I extend my deepest gratitude to Chris, Emma, and Helen for making it all worthwhile

Alastair D. Reith

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Cloning Protein Tyrosine Kinases by Screening cDNA Libraries with Antiphosphotyrosine Antibodies

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1. Introduction

Protein tyrosine kinases (PTKs) play prominent roles in the regulation of fundamental biological processes including normal cell growth and survival, cell differentiation, and development. Across vertebrate and invertebrate species, both nonreceptor (cytoplasmic) and receptor (transmembrane) type PTKs have been identified, making them one of the most extensively examined family of proteins. Currently, genes encoding at least 50 receptor and 33 nonreceptor vertebrate PTKs have been cloned ***(1,2)***, several by techniques that exploit the structural and functional conservation of the kinase catalytic domain.

The catalytic domain of PTKs is comprised of approx 250 amino acids that can be divided into 11 highly conserved sequence motifs ***(3)***. This homology has been successfully utilized in the molecular-based cloning of novel and known PTKs. These stratagies have included the low stringency screening of cDNA libraries with probes homologous to the catalytic domain of preexisting PTK clones, the use of degenerate oligonucleotides as hybridization probes, and the use of degenerate oligonucleotides as primers for polymerase chain reaction (PCR)-based screening.

In addition to sequence similarity, the kinase domains of PTKs possess phosphotransferase activity, making them functionally related. The transphosphorylation and autophosphorylation activities of PTKs have been well documented ***(1,2)*** and interestingly, the expression of just the catalytic domain in *Escherichia Coli* results in an active tyrosine kinase ***(4,5)***. The technique of

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detecting protein phosphorylation on tyrosine residues by immunoblotting with phosphotyrosine-specific antibodies has proven highy sensitive in Western blot analysis ***(6)***. Because endogenous PTK activity in bacteria is negligible, the premise on which expression cloning functional PTKs is based is the use of antiphosphotyrosine antibodies to detect active tyrosine kinases that are expressed from cDNA clones introduced into bacteria.

The main advantage of this functional screening approach is confirmation of the catalytic activity of the cloned PTK gene. This method also allows for the potential cloning of novel kinases that phosphorylate tyrosine residues, because there is no sequence bias in this procedure. PTK cDNAs that diverge from the normal sequence would not be found by nucleic acid hybridization techniques. Moreover, this functional screen has facilitated the identification of an emerging family of dual specificity protein kinases that phosphorylate serine, threonine, and tyrosine residues ***(7–9)***.

The use of antibodies in general to screen expression libraries has been described previously ***(10,11)***, and modifications suitable for the use of antiphosphotyrosine antibodies will be described here. The most important aspects of this procedure are the possession of an expression library that is ready to screen and an antiphosphotyrosine antibody, either of which can be commercially obtained or generated according to already published procedures ***(12–14)***. For simplicity, we will describe the use of a lambda gt11 cDNA expression library, which is commonly used for immunological screening. However, other types of equally suitable expression libraries will be described below.

In principle, a lambda *gt11* cDNA expression library allows for the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible production of cDNA-encoded proteins fused to β-galactosidase in bacteria. A simple procedure is used for plating libraries on an expression host, which results in a single plaque arising from a single phage that has infected one bacterium (*see* **Note 1**). An active tyrosine kinase produced could phosphorylate itself and bacterial proteins on tyrosine, and this is detectable on nitrocellulose filters probed with antiphosphotyrosine antibodies. Positive clones are visualized by either radioactive or nonradioactive methods. A cDNA encoding a potential PTK is further characterized molecularly and biochemically to confirm its identity. The efficacy of this approach has been demonstrated in the cloning of both nonreceptor and full-length receptor tyrosine kinases ***(15–20)***.

2. Materials

2.1. The Bacteriophage Lambda cDNA Expression Library

Optimally, the library should contain inserts no smaller than 1.0 kb, which is the minimal size required to comprise the catalytic domain of a PTK ***(3,15)***. Several innovative modifications of the conventional lambda *gt11* cDNA ex-

pression library are now available, as well as other bacteriophage lambda libraries such as Lambda Zap (Stratagene) and Lambda EXlox (Novagen). These libraries contain features that not only increase the efficiency of detecting positive clones, but also eliminate the need to eventually subclone cDNA from lambda into prokaryotic or eukaryotic vectors. These features include an increased cloning capacity and unidirectional cloning of cDNA into lambda, and in vivo excision systems of cDNA from the recombinant lambda DNA. In addition, for biochemical analysis of a cloned PTK, the cDNA may be subcloned into vectors which allow its expression as a fusion protein with an epitope tag (*myc*, *T7*, *HA*, *His*) to which antibodies are commericially available ***(21)***. Alternatively, the vector insert in lambda may itself contain sequences encoding either an epitope tag or glutathione S-transferase ***(22)***, and the cloned PTK may be expressed as a fusion protein from the plasmid on its in vivo excision and isolation from lambda DNA. We recommend consideration of other library constructions that contain these additional features.

2.2. Antiphosphotyrosine Antibodies

The production of effective polyclonal antibodies (PAb) and monoclonal antibodies (MAb) that recognize phosphotyrosine (PY) has a rich history, and although commercially available, procedures for their generation have been well documented ***(12–14)***. Anti-PY antibodies have been raised against a variety of antigens including phosphotyrosine, structural analogues such as phosphotyramine or p-aminobenzylphosphonic acid, polymerized mixtures of phosphotyrosine, alanine and glycine/threonine, and the bacterially expressed catalytic domain of the PTK v-abl. These antibodies are broadly reactive and recognize phosphorylated tyrosine in the context of many peptide sequences. Alternatively, it is possible to produce polyclonal antiphosphopeptide antibodies that recognize a specific PTK in its phosphorylated state. An oligopeptide containing a phosphorylated tyrosine residue can be synthesized based on the tyrosine phosphorylation site in the PTK of interest ***(23)***. This strategy was successfully employed to generate antibodies to the tyrosine-phosphorylated form of the PTK neu ***(24)***. Ultimately, you need a stock of anti-PY antibody that will detect tyrosine-phosphorylated proteins by Western blot analysis (*see* **Note 2**).

Some preliminary tests of your stock anti-PY antibody are recommended and are relatively simple. For a commercial antibody, Western blot analysis of a cell lysate that contains tyrosine-phosphorylated proteins of known molecular weights (this is also commercially available), under the conditions you will use to screen the library, will verify its specificty and effective concentration. It is assumed that anti-PY antibodies generated yourself have been extensively characterized already. In general, a concentration of 1–3 µg/mL is suitable for

Western blots and library screening. Because serum often contains anti-*E. Coli* reactive antibodies, these can be removed before you begin library screening by presorption onto *E. Coli* protein lysates. *E. Coli* protein-coated filters (*see* **Subheading 3.1.1.**), obtained from plated lambda *gt11* phage that do not contain inserts, can be reacted with anti-PY serum under library screening conditions. It may be necessary to do this several times, but the same lambda *gt11* plate can be used to produce several filters. You will have a cleaner antibody to screen with as a result. In general, MAbs have less background reactivity with *E. Coli* proteins.

Because not all tyrosine-phosphorylated proteins that bind to monoclonal anti-PY antibodies bind to polyclonal anti-PY antibodies ***(14)***, MAbs may have a lower binding constant, and sensitivity may be compromised. However, specific signals detected by a monoclonal anti-PY antibody may be amplified by altering the secondary detection reagent (*see* **Subheading 3**). Finally, although PAbs may be reused several times, we do not suggest this as their properties may change with reuse (*see* **Note 3**). In contrast, MAbs can be reused several times. For storage and reuse of antibodies, sodium azide should be added to 0.02% (*see* **Note 4**). Antibodies can be kept at 4°C for up to 1 mo and used 5–10 times.

2.3. Reagents

2.3.1. Library Plating and Plaque Isolation

1. *E. coli* strain Y1090 (Stratagene, genotype: Ä(lac)U169 araD139 strA supF mcrA trpC22::Tn10 (Tet^r) [pMC9 Amp^r Tet^r]).
2. LB media: 10 g/L bactotryptone, 5 g/L yeast extract, 5 g/L NaCl, final pH 7.5. Autoclave, then add filter sterilized 1 *M* $MgSO_4$ to 10 m*M* final concentration.
3. Ampicillin: 100 mg/mL in distilled water, sterile filtered with Millipore 0.22 µm filter, and stored at –20°C.
4. 10% (w/v) maltose in distilled water. Sterile filtered and stored at 4°C.
5. 10 cm and 15 cm Petri dishes.
6. Bottom agar: 15 g agar in 1 L LB, autoclaved. Used to make LB plates.
7. Top agarose: 0.75 g agarose in 100 mL LB, autoclaved.
8. Phage buffer: 0.1 *M* NaCl, 0.05 *M* Tris base, 0.1% gelatin, final pH 7.5, autoclaved. Add sterile-filtered $MgSO_4$ to a final concentration of 10 m*M*.
9. T *M* buffer: 20 m*M* Tris-HCl, pH 8.0. Autoclave and add sterile-filtered $MgSO_4$ to a final concentration of 10 m*M*.
10. Chloroform.
11. 1 *M* $CaCl_2$, autoclaved.
12. 10 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG): Made in double-distilled water and stored at –20°C.
13. 10% sodium azide: Made in double-distilled water and stored at room temperature.

14. Tris-buffered saline (TBS): 0.17 *M* NaCl, 0.01 *M* Tris base, final pH 7.5.
15. TBST: TBS with 0.05% Tween-20.
16. 1 *M* $MgSO_4$: sterile filtered with Millipore 0.22 µm filter and stored at room temperature.
17. Nitrocellulose filters for 10 and 15 cm Petri dishes (Stratagene #420106 and #420107).
18. Sterile Pasteur pipets.
19. Sterile 10–15 mL glass or polypropylene tubes.
20. Markers: Syringe needle (Becton-Dickinson 20G needle, #305175), water insoluble ink pen (VWR Scientific Products #52877-150), or fluorescent markers (VWR Scientific Products #52878-180).

2.3.2. Screening Filters

1. Blocking Solutions:
   a. Block Type A: 5% bovine serum albumin (BSA) (Sigma #A-2153) in TBST.
   b. Block Type B: 5% BSA and 1% ovalbumin (Sigma #A-5503) in TBST.
   c. Block Type C: 20% fetal calf serum (heat inactivated) (Gibco-BRL #16000-036) in TBST.
   d. Block Type D: 2% goat serum (heat inactivated) (Gibco-BRL #16210–064), 1% fish gelatin (Norland Products), and 1% BSA in TBST.
2. Secondary screening reagents (*see* **Note 5**) ***(25)***:
   a. Radioactive: ^{125}I coupled to protein A or protein G (30 mCi/mg specific activity) (NEN; Amersham; ICN) or coupled to an appropriate secondary antibody, X-ray film, intensifying screen, Saran wrap.
   b. Nonradioactive:

      Type A: Horseradish peroxidase (HRP) coupled to protein A (Boehringer-Mannheim #605-295 at 1:5000) or protein G (Bio-Rad #170-6467) or coupled to an appropriate secondary antibody (Boerhinger-Mannheim #1814-141 at 1:20,000 dilution and #1812-168 at 1:10,000 dilution). Visualization solution is comprised of 5 mL of 100 m*M* Tris-HCl, pH 7.5 containing 100 µL of DAB (40 mg/mL of 3,3'-diaminobenzidine in H_2O), 25 µL $NiCl_2$ (80 mg/mL in H_2O), and 15 µL of 3% H_2O_2. Solutions are also commercially available (Pierce; Biorad; Boerhinger-Mannheim). Alternatively, other chromogenic substrates may be used, and are described elsewhere ***(26)***. For enhanced chemiluminescence (ECL)-based detection, commercial kits are available (Amersham; Pierce; Bio-Rad; Boerhinger-Mannheim) in which equal volumes of luminol reagent and oxidizing agent are mixed for use. Otherwise, ECL visualization solution can be made by mixing 0.5 mL of 10× luminol solution (4 mg luminol/mL dimethyl sulfoxide [DMSO]), 0.5 mL 10× p-iodophenol stock (10 mg/mL in DMSO), 2.5 mL of 100 m*M* Tris-HCl, pH 7.5, and 25 µL of 3% H_2O_2, in a 5-mL final volume (with H_2O).

      Type B: Alkaline phosphatase (AP) coupled to protein A (Boerhinger-Mannheim #100-052 at 1:1000), or coupled to an appropriate secondary antibody (Boerhinger-Mannheim #1814-206 at 1:5000 and #1814-214 at 1:5000).

For chromogenic detection, AP buffer consists of 100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, and 5 m*M* $MgCl_2$. BCIP/NBT visualization solution is comprised of 5 mL of AP buffer containing 33 µL of NBT (50 mg/mL of 5-bromo-4-chloro-3-indolyl phosphate in 70% dimethyl formimide) and 17 µL of BCIP (50 mg/mL of nitroblue tetrazolium in 100% dimethyl formimide).

Type C: Biotinylated secondary antibody (Boerhinger-Mannheim #605-100 at 1:1000 and #605-195 at 1:15,000; Bio-Rad #170-6401) and avidin conjugated to alkaline phosphatase (Boerhinger-Mannheim #100-200 at 1:2500; Bio-Rad #170-6533) or HRP (Bio-Rad #170-6528).

2.3.3. Clone Identification

1. dNTPs (Boerhinger-Mannheim #104035, #104094, #104272).
2. α-^{32}P-dATP (NEN #Blu-Neg512H).
3. DNAse I (Stratagene #600031).
4. DNA polymerase I (Boerhinger-Mannheim #642711).
5. 0.5 m*M* ethylenediaminetetracetic acid (EDTA) (autoclaved).
6. tRNA (Boerhinger-Mannheim #109495).
7. TE buffer: 10 m*M* Tris-HCl and 1 m*M* EDTA, pH 8.0, autoclaved.
8. Phenol (Ambion #9730).
9. Sephadex G-50.
10. Klenow fragment of *E. coli* DNA polymerase I (Stratagene #600071).
11. Nitrocellulose membrane (Stratagene #420115).
12. Base denaturing solution: 1.5 *M* NaCl and 0.5 *M* NaOH.
13. Neutralization solution: 1 *M* NaCl and 0.5 *M* Tris-HCl, pH 7.0.
14. Hybridization solution: 5× SSC, 5× Denhardt, 1% SDS, and 100 µg/mL denatured salmon sperm DNA.
15. 20× SSC solution: 3 *M* NaCl and 0.3 *M* Na_3 Citrate, pH 7.0.
16. 100× Denhardt solution: 2 g/L ficoll, 20 g/L polyvinylpyrrolidone, 20 g/L BSA.
17. 0.4 *M* NaOH.
18. Wash solution: 200 m*M* Tris-HCl, pH 7, 0.1× SSC,and 0.1% SDS.
19. Denatured salmon sperm DNA (Stratagene #201190).

2.3.4. Kinase Activity Analysis

1. 4× Laemmli reducing sample buffer: 0.25 *M* Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, and 0.25% bromophenol blue. Store at 4°C.
2. Lysis buffer: 1% Triton X-100, 150 m*M* NaCl, 10% glycerol, 1 m*M* sodium orthovanadate, 2 m*M* EDTA, and 20 m*M* Tris-HCl, pH 7.5.
3. Ripa buffer: lysis buffer including 0.1% sodium dodecyl sulfate (SDS) and 0.5% sodium deoxycholate.
4. γ-^{32}P-ATP (NEN #BLU-NEG502A).
5. Kinase buffer: 20 m*M* HEPES, pH 7.5, 10 m*M* $MgCl_2$, and 10 m*M* $MnCl_2$.
6. Protein A-Sepharose (Pharmacia Biotech #17-0780-010).
7. GammaBind G-Sepharose (Pharmacia Biotech #17-0885-01).

8. 10× Phosphate-buffered saline (PBS), calcium- and magnesium-free (Gibco-BRL #70011-044).
9. ^{32}P-orthophosphate (NEN #NEX053S).
10. M9 media: 0.5% casamino acids, 0.1 m*M* $CaCl_2$, 0.02% glucose, 10 µg/mL thiamin, 6 g/L Na_2HPO_4-$7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, and 1 g/L NH_4Cl; add sterile filtered $MgSO_4$ to 1 m*M* final concentration.

3. Methods

3.1. Screening the Expression Library with Antiphosphotyrosine Antibody

3.1.1. Plating the Library for Screening

1. A culture of *E. coli* Y1090 should be grown overnight at 37°C, with moderate shaking (2500 rpm), in LB media containing 50 µg/mL of ampicillin and 0.2% maltose. Subsequently, this culture may be stored at 4°C and used later to grow cultures (*see* **Note 6**).
2. For each 15 cm LB plate, 1 mL of the *E. coli* Y1090 overnight culture is centrifuged (4000*g*, 15 min, 4°C) and the bacterial pellet is resuspended in 0.5 mL of T *M* buffer, or in 10 m*M* $MgSO_4$.
3. The bacterial suspension is then infected with 1×10^4 to 5×10^4 lambda *gt11* recombinant phage for 15 min at 37°C. We found 2×10^4 pfu/plate to be convenient. Use phage buffer to make appropriate dilutions of the stock lambda *gt11* library (*see* **Note 7**).
4. Meanwhile, prewarm the 15-cm LB plates at 42°C. Each plate should have an identification mark on its base (*see* **Note 8**).
5. Top agarose should be well dissolved and kept at 45–50°C.
6. For each 15-cm LB plate, 5 mL of top agarose is removed into a sterile tube and the infected Y1090 bacteria is added. Mix gently and quickly pour onto the LB plate, without forming air bubbles. This is best achieved by pouring the agarose-cell suspension along the inside wall of the agar plate and gently shaking plate in a cirular motion on the bench top to get an even overlayer. Let the plate set for 5 min with the lid slightly off.
7. Incubate the plates, inverted, for 3 to 5 h at 42°C, or until clear plaques, of approx 1 mm in diameter, are detectable.
8. Meanwhile, soak nitrocellulose filters in 10 m*M* IPTG (*see* **Note 9**). Let them air-dry on Saran Wrap™. Wear gloves and use forceps to handle the filters. When dry, mark each filter with a water insoluble ink marker to correspond to its LB plate.
9. Carefully overlay each LB plate with an IPTG-impregnated filter. Do not form air bubbles. This is best accomplished by bending the filter in the center and placing the midline of the filter in the middle of the plate. Then slowly allow the filter to make contact with the agarose surface. Do not lift and move the filter once it has contacted the surface. Incubate plates, inverted, at 37°C for 8–10 h. Reasonable protein expression occurs by 4 h.

10. Before removing the filter, mark its position on the LB plate by poking small holes in an assymetric pattern through the filter and into the agar. You can turn the plate over and mark where the holes are on the base of the plate with a pen.
11. Carefully, lift the nitrocellulose off without removing the top agarose. If top agarose sticks to the filter, cool plates at 4°C for 15 min before lifting the filter. Place the filter, agarose-contact side up, into a Petri dish containing TBS. For duplicate screening, a second IPTG-impregnated filter may be placed on the plate for 4 h to overnight at 37°C (*see* **Note 10**). Remember to mark the second filter in the same places as the first.
12. Rinse the filters four times in TBS, 10 min each time at room temperature, with gentle rocking.
13. If filters are not to be screened immediately, the last wash should be done with TBST containing 0.02% sodium azide. Filters can be stored in individual Petri dishes with TBST/azide at 4°C, or they can be air dried on Saran Wrap™, wrapped, and stored at toom temperature. The LB plates with plaques may be wrapped in parafilm and stored at 4°C.

3.1.2. Screening Filters with Antiphosphotyrosine Antibodies

1. After a final wash in TBST, filters are incubated with 15 mL of blocking solution (Types A–D; *see* **Subheading 2.3.2.**) with gentle rocking, for at least 2 h at room temperature or overnight at 4°C (*see* **Note 11**). Block type D is the most effective for reducing background signals.
2. Remove blocking solution and wash once in TBST for 10 min.
3. Incubate filters with 10–12 mL of blocking solution containing antiphosphotyrosine (PY) antibody (1–3 µg/mL) for at least 2 h at room temperature or overnight at 4°C, rocking gently (*see* **Note 12**). If screening a large number of filters ***(30–40)***, a container with a diameter slightly larger than the filter should be used to conserve volume. A 2-L beaker with 15-cm filters that are individually separated by nylon mesh works well.
4. Filters are then washed four times in TBST, 10 min each time at room temperature, with gentle rocking. Bound anti-PY antibodies can be detected on the filter by either a radioactive method (*see* **step 5A**) or by a nonradioactive method (*see* **step 5B**).

5A. Radioactive detection: Treat filters with 10–12 mL of blocking solution containing ^{125}I-labeled protein A or an ^{125}I-conjugated secondary antibody (at approx 0.1–0.5 µCi/mL) for at least one hour at room temperature (or 4°C overnight), with gentle rocking. Filters should then be transferred to a new Petri dish and washed four times in TBST as in **step 4**, above. Filters are then air-dried on Saran Wrap™, wrapped in Saran Wrap™, and marked with a radioactive pen or fluorescent marker for later alignment. The filters should be exposed to X-ray film under an intensifying screen at –70°C for a few days before developing.

5B. Nonradioactive detection: Treat filters with 10–12 mL of blocking solution containing HRP or AP conjugated to either protein A or protein G, or to an appropriate secondary antibody, using a dilution either recommended by the

manufacturer or previously determined by Western blot analysis (*see* **Subheadings 2.2.** and **2.3.2.**) for 1 h with gentle rocking. Filters should then be transferred to a new Petri dish and washed four times in TBST as described in **step 4**. An appropriate substrate is then added for chromogenic or chemiluminescence detection (*see* **Note 13**).

a. AP-based assay: Rinse the blot in AP buffer (100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$). Add BCIP/NBT visualization solution and rock gently. When staining is apparent (indigo/dark blue color in 10 to 30 min), stop the reaction by washing the filter several times with water and air-dry.
b. HRP-based assay: Add DAB/$NiCl_2$ visualization solution to filter and rock gently. When staining is apparent (dark brown), wash the filter several times with water and air-dry. Alternatively, other chromogenic substrates may be used (*see* **Subheading 2.3.2.**) ***(26)***.
c. ECL-based assay: If using a commercial kit (Amersham; Pierce; Bio-Rad; Boerhinger-Mannheim), mix equal volumes of the luminol reagent and oxidizing agent. Otherwise, ECL visualization solution can be made as described (*see* **Subheading 2.3.2.**). Add to filter and agitate gently for 1 min. Drain excess liquid from filter before wrapping in saran or placing between clear plastic sheets. Mark plastic or saran with a fluorescent marker for later alignment. Expose to X-ray film in the dark for 5 s to 30 min. Develop film.
d. Biotinylated secondary antibody: Add biotinylated secondary antibody to the filter for 1 h with gentle rocking. Wash the filter three times with TBST as in **step 4**. Transfer into TBST containing an avidin-HRP complex or an avidin-AP complex for 30 min with gentle rocking. Wash the filter three times with TBST as in **step 4**. Visualize by the addition of an appropriate chromogenic substrate as described in **steps a–c**. This procedure amplifies the signal derived from a single plaque, but may also increase background staining as well.

3.1.3. Isolation and Rescreening of Positive Plaques

1. Once positive plaques have been identified, the filters or X-ray film should be matched to their corresponding LB plates.
2. The large end of a sterile Pasteur pipet can be used for removing agar plugs that contain the positive phage by stabbing it through the top agarose into the hard agar beneath.
3. The agar plug is released by shaking the pipet end into a sterile tube containing 1 mL of phage buffer and one drop of chloroform. Let phage particles diffuse out for 1–2 h at room temperature. This phage stock solution may be stored at 4°C. Typically, a plaque has 10^6–10^7 infectious particles.
4. Each phage stock solution is diluted 10^2–10^4 in phage buffer and each dilution is plated onto an LB plate as described above (*see* **Subheading 3.1.1.**, **steps 1–6**).
5. Filters are screened again with anti-PY antibody as described above (*see* **Subheading 3.1.1.**, **steps 7–13**). A third rescreen is done on 10-cm LB plates for the final isolation of single positive phage.

3.2. Molecular Analysis of Positive Clones

3.2.1. Isolation of Recombinant Bacteriophage Lambda DNA from Positive Plaques

1. A Y1090 culture is grown overnight at 37°C in LB containing maltose and ampicillin as described in **Subheading 3.1.1.**, **step 1**.
2. Bacteria from 1 mL of culture is pelleted (4000*g*, 15 min, 4°C) and resuspended in the same volume of T *M* buffer. This is infected with 10^6- 10^7 phage particles from a purified single phage stock, for 30 min at room temperature.
3. Infected bacteria is then transferred to 40 mL of LB containing 5 m*M* $CaCl_2$ and 50 µg/mL ampicillin, and the culture is shaken vigorously for 1 h at 37°C.
4. Bacteria from 20 mL is pelleted (4000*g*, 15 min, 4°C).
5. Recombinant bacteriophage can be purified from the bacterial pellet using a commercially available preparatory kit (Stratagene, Promega) according to the manufacturer's instructions (*see* **Note 14**).

3.2.2 Identification of Independent Clones

1. If you have many positive clones, we recommend that they first be classified according to their cDNA inserts. Inserts may be excised from recombinant lambda *gt11* phage DNA by restriction enzyme digestion, and subsequently subcloned into prokaryotic or eukaryotic expression plasmids for large scale propagation and further molecular analysis. As mentioned above (Section 2.1), some libraries offer a convenient in vivo excision system of plasmids from the recombinant lambda phage, which eliminates the need to subclone.
2. Cross-hybridization analysis: This method may be useful if a large number of phage are isolated, to quickly determine that recombinant phage harbor different genes. cDNA inserts can be used to make radioactive or nonradioactive probes for use in a hybridization procedure which screens all of the positive recombinant lambda phage DNA with each cDNA insert ***(27)***. cDNA inserts may be labeled with ^{32}P by either nick translation or random oligonucleotide primed synthesis (*see* **Note 15**) ***(28)***. Screening of lambda phage DNA can be accomplished using a dot blot technique ***(29)*** in which the recombinant phage DNA are immobilized on a nitrocellulose or nylon membrane for hybrization with each probe ***(30)***. Clones that hybridize with a single probe are scored as different isolates or different portions of the same gene. Filters can be washed and sequentially reprobed with each cDNA insert probe.
   a. Labeling cDNA by nick translation: Mix 0.25 µg of a gel-purified fragment of the cDNA (100–1000 bases long) with 2.5 µL of 0.5 m*M* 3dNTP mix (no dATP), 100 µCi of $\alpha^{32}P$-dATP, 1 µg of DNAse I, and 1 µL of DNA polymerase I (25 µL final volume), and incubate at 14°C for 30–45 min. Stop the reaction by adding 1 µL of 0.5 m*M* EDTA, 3 µL of tRNA (10 mg/mL stock), and 100 µL of TE buffer. Phenol extract the mix and apply the aqueous (top) phase to a Sephadex G- 50 column to remove unincorporated nucleotides. The specific activity of the probe should be approx 10^8 cpm/µg.

b. Labeling cDNA by random oligonucleotide primed synthesis: Mix 100 ng of gel-purified cDNA that has been heat-denatured (100°C for 10 min, then chilled on ice) with 1 ng of random sequence hexanucleotides as described in **step2a** above, but substitute Klenow for DNAse I and DNA pol I. Phenol extract, and purify as described in **step2a**.
c. Dot blotting the cDNA: This can be done manually, but a vacuum/manifold device gives the most consistent results. Heat-denature DNA (100°C for 10 min, then chilled on ice) or base denature DNA (2 µL DNA in 100 µL of 1.5 *M* NaCl/0.5 *M* NaOH at 37°C for 20 min) and apply to the nitrocellulose (or nylon) membrane (in "dots"). Place the membrane in a glass dish and treat for 10 min in denaturing solution and then for 10 min in neutralization solution. If using nitrocelluose, bake the membrane for 2 h at 80°C to immobilize the DNA. If using a nylon membrane, immobilize the DNA by crosslinking with ultraviolet (UV) light. We recommend using a modified nitrocelluose membrane (Stratagene) which combines the strength of nylon with the lower background of nitrocelluose.
d. Hybridization analysis: Treat the dot blot with 6¥ SSC and then hybridization solution for 3 h at 55–68°C (use a heat-sealable polyethylene bag, or use a hybridization bottle for a rotary style oven). Add hybridization solution containing the ^{32}P-labeled probe (2.5×10^5–1×10^6 cpm/mL) and incubate overnight at 55–68°C. There are also commercially available quick hybridization solutions that allow hybridization in 1–2 h (Stratagene). Wash in 2× SSC/0.1% SDS and 0.2× SSC/0.1% SDS in succession at 55–68°C. Air dry the blot and expose to film. The dot blot may be stripped in either boiling water for 5 min or in 0.4 *M* NaOH for 30 min at 45°C. Wash the blot with wash solution at room temperature with gentle agitation before reprobing.

3. Restriction Enzyme Digestion Analysis: Alternatively, once inserts are subcloned into a plasmid, restriction enzyme mapping can be used to identify identical and independent clones.

3.2.3. Sequence Identification of Cloned PTKs

1. Sequence analysis is the most direct method to identify a cloned PTK. If few positive clones are identified in the library screen, then the insert cDNA sequences can be subcloned and sequenced immediately. Once multiple cDNAs have been categorized into groups that represent single clones, the cDNA from one member of each group can be sequenced for identification. Using primers specific to the lambda *gt11* vector, one can sequence without subcloning. Such primers are commercially available (Clontech). Finally, the nucleic acid and the translated amino acid sequence can be compared to those deposited in sequence databases (GenBank, EMBL) and be identified as known or novel.
2. While full-length receptor-type PTKs have been successfully isolated from a single positive clone, it is possible that you will isolate only a partial cDNA clone of a PTK. It will then be necessary to use the partial cDNA to screen a nucleic acid library ***(18,31)***. If the 5-prime end of the clone is missing, you may try using

5-prime rapid amplification of cDNA ends (RACE) ***(32)*** to isolate the missing part of the gene.

3.3. Functional Analysis of Positive Clones

3.3.1. Analysis of Bacterial Lysates

Expression of a cloned PTK cDNA in bacteria is a convenient way to verify its kinase activity since there is no bacterial background PTK activity ***(7)***. After protein synthesis is induced, bacterial lysates can be examined biochemically by Western blot analysis using an anti-PY antibody and an antibody to the protein that is fused to the PTK (such as anti-β-galactosidase antibody). The PTK-fusion proteins are usually the proteins most heavily tyrosine-phosphorylated in lysates because of autophosphorylation.

1. Induce protein expression either in bacteria that harbor the recombinant bacteriophage lambda, or in bacteria that have been transformed with an expression plasmid containing the cDNA insert from the bacteriophage, under the appropriate conditions. For example, grow transformed bacteria by shaking (2500 rpm) overnight at 37°C in 3 mL of LB media with the appropriate antibiotic (such as 50 µg/mL ampicillin). Dilute the overnight culture 1:100 in LB media, grow for 2–3 h at 37°C with shaking, and then induce protein expression for 2–3 h at 37°C by adding the appropriate agent (addition of IPTG to 10 m*M* final concentration for example). If inducing expression from bacteria infected with a recombinant phage, grow and infect bacteria as in **Subheading 3.2.1.**, **steps 1–3**. Protein expression is induced by adding IPTG (10 m*M* final concentration) and shaking the culture for another 2–3 h.
2. Bacteria are harvested by centrifugation at 4000*g* for 10 min at 4°C.
3. The bacterial pellet is lysed by resuspending in 1× PBS (0.5 mL of 1× PBS for 1 mL of bacterial culture) and sonicating with a microprobe-equipped sonicator, or by freezing on dry ice and then thawing. Lemmli sample buffer is added to a 1¥ final concentration and the sample is boiled for 2 min. Samples may be examined by Western blot analysis (10–20 µL out of a 500-µL sample is sufficient) immediately, or frozen at –20°C.
4. Alternatively, the cloned PTK-fusion protein may be immunoprecipitated from the bacterial pellet for Western blot analysis. After protein expression is induced, bacteria can be pelleted and resuspended in ice-cold lysis buffer that does not contain 1% Triton X-100. Sonicate or freeze/thaw the suspension to lyse the bacteria as described in **Subheading 3.3.1.**, **step 3**. Add Triton X-100 to a final 1% concentration and mix thoroughly. Clear the lysate by centrifugation at 10,000*g* for 5 min at 4°C. Adjust the lysate supernatant to 0.1% SDS and 1% sodium deoxycholate for RIPA conditions, if desired. Add the precipitating antibody (approx 1–5 µg) and incubate for 4 h to overnight, at 4°C, with gentle rotation. Add protein A or protein G conjugated to

Sepharose for 1 h at 4°C with rotation. Pellet the Sepharose by brief centrifugation and wash three times with either lysis buffer or RIPA buffer. Resuspend the Sepharose in 1× Lemmli sample buffer and boil for 2 min. The samples may be analyzed immediately or frozen at –20°C.

3.3.2 Kinase Activity Analysis

As described in **Subheading 3.3.1.**, PTK-fusion proteins can be immunoprecipitated with either an anti-PY antibody or with an antibody to the protein that is fused to the PTK. This immunocomplex can be subjected to an in vitro kinase reaction using γ-^{32}P-ATP to validate its identity as a PTK.

1. Following immunoprecipitation, the complex is washed three times with ice-cold lysis buffer or RIPA buffer, and then two times with kinase buffer.
2. Resuspend the complex in 50 µL to 100 µL of kinase buffer containing 5–50 µCi of γ-^{32}P-ATP (greater than 5000 Ci/mmole specific activity) and incubate at 30°C for 20 min.
3. Add 4× Lemmli sample buffer to 1× final concentration and incubate at 100°C for 2 min. Samples can be analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography to functionally validate its identity as a PTK.

Alternatively, the PTK-fusion protein that is expressed in bacteria can be metabolically labeled in vivo with ^{32}P-orthophosphate and subjected to phosphoamino acid analysis to verify autophosphorylation on tyrosine ***(33)***. This method is labor intensive and is probably more beneficial when dual kinase activity is suspected ***(7)*** (*see* **Note 16**).

1. After protein expression is induced, pellet the bacteria by centrifugation at 4000*g* for 10 min at 4°C. Resuspend the pellet in M9 media (0.1 mL media for each 10 mL of the original bacteria culture) containing 500 µCi of ^{32}P-orthophosphate, and incubate for 30 min at 37°C. You can also induce expression and label proteins with ^{32}P-orthophosphate simultaneously in M9 media, for several hours to overnight.
2. Collect bacteria by centrifugation at 4000*g* for 10 min at 4°C, wash three times with M9 media by resuspending and pelleting, and lyse as described in **Subheading 3.3.1.**, **step 3**.
3. Alternatively, the ^{32}P-labeled PTK-fusion protein can be immunoprecipitated as described in **Subheading 3.3.1.**, **step 4**.
4. For phosphoamino acid analysis, lysates or immunoprecipitates containing the ^{32}P-labeled PTK fusion protein can be resolved by SDS-polyacrylamide gel electrophoresis and isolated either directly from the gel, or transferred and immobilized onto nitrocelluose for isolation. The isolated protein is then subjected to acid hydrolysis and products resolved by two-dimensional electrophoresis on cellulose thin-layer plates (*see* **Note 16**).

4. Notes

1. The genetics and lytic cycle of bacteriophage lambda will not be described here and we refer you to a detailed description elsewhere ***(34)***.
2. Commercially available anti-PY antibodies: rabbit polyclonal (UBI) and mouse monoclonal (PY20: ICN, Zymed, Transduction Laboratories; 4G10: UBI).
3. For Western blots, staining of protein bands may become more or less prominent with each reuse of anti-PY antibodies. This may be because of the loss of high-affinity antibodies during early uses ***(25)***.
4. Sodium azide is toxic.
5. Reagents for chromogenic detection based on HRP or AP are available as commercial kits (Pierce; Bio-Rad; Boerhinger-Mannheim), as are the ECL reagents (Amersham; Pierce; Bio-Rad; Boerhinger-Mannheim).
6. When using specialized bacteriophage cDNA expression libraries, it may to necessary to use specific bacterial host strains and conditions for plating.
7. Because it is desirable to have space between individual plaques, you may need to try plating several different dilutions of the stock lambda *gt11* library to determine a reasonable plaque density.
8. It is best to pour LB plates 2–4 d in advance and store them inverted at room temperature. Try to avoid condensation formation in the Petri dish and on the lid, as moisture may accumulate on the top agar and cause plaques to streak together. Moisture can be absorbed carefully with filter paper.
9. Nitrocellulose should be initially moistened according to the manufacturer's recommendations.
10. Plates are often screened in duplicate for the primary screen in order to avoid false positives.
11. A variety of blocking solutions have been successfully employed. Do not use nonfat dry milk in the blocking solution, because it contains constituents which bind to anti-PY antibodies.
12. Most antibodies produce a good signal at room temperature. Incubation times can be varied and in general, 2–4 h is good. An 8–10 h incubation may give you a signal that is up to ten times stronger. If you are using a low-affinity anti-PY antibody, then incubate filters overnight with antibody at 4°C.
13. If an ECL-based detection system is used, do not use sodium azide in any solutions, as it interferes with chemiluminescence chemistry. HRP and AP catalyze the formation of insoluble colored precipitates directly on the surface of the filter and positive plaques may be located more accurately than by X-ray film. The signal produced by AP remains active slightly longer than that produced by HRP.
14. Lambda DNA purification kits offer rapid methods which produce high quality DNA for restriction enzyme digestion, cDNA insert mapping, and sequencing. However, a detailed description of bacteriophage lambda DNA isolation using polyethylene glycol (PEG) precipitation and phenol/chloroform extraction is available ***(34)***.

15. For nonradioactive alternatives using biotin and digoxigenin, and detection by AP, HRP, ECL, and immunoflourescence, *see* **ref. *35***.
16. Phosphoamino acid analysis is described in Chapter 4.

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