

Detection, Isolation and Sequence Comparisons of Protein Kinase Genes

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Abstract

This is a research exercise involving the isolation of genes for tyrosine kinases and comparison of their partial DNA sequences. Genes for tyrosine kinases expressing active enzymes in bacteriophage expression libraries can be detected using anti-phosphotyrosine antibodies. This is based on the observation that there is normally no detectable phosphorylation of tyrosine in bacterial proteins but, when an active tyrosine kinase is expressed in bacteria, the phosphorylated proteins can be detected by antibodies using an enzyme linked antibody assay system. *E. coli* bacteriophage expression libraries from a variety of sources can be used for the experiments. The isolated clones can be partially sequenced using standard primers and chemiluminescent sequencing or can be sent to commercial laboratories to be sequenced. Sequences can be compared to each other and to sequences in existing databases. Students work cooperatively in groups along with the instructor and the groups gather to discuss progress, compare sequences and then present their results at the end of the project. Potentially, groups from different schools could submit their sequences to a common database where they could be accessed and compared by the other groups. Students have opportunity to consider the various roles kinases play in cell functions in a variety of organisms, to work with immunological and molecular genetic techniques and to analyze and compare original data.

The Process of Science in Introductory Biology Labs

Wheaton College is a primarily undergraduate institution with approximately 2400 undergraduates mainly from evangelical Christian backgrounds. Admission to the school is highly competitive and the students are therefore a very capable and enthusiastic group. Interactions with these students was, in fact, one of the reasons I recently decided to move, after doing research and teaching at the University of Pennsylvania School of Medicine for the past 20 years, to Wheaton to teach Biology to undergraduates. Having made this transition, I could not help comparing the experiences I had doing research with the impression of laboratory work that the freshmen were receiving from their introductory Biology courses.

Instead of viewing laboratories as places where you solve interesting puzzles by asking and working out ways to answer a series of questions, I found that students tended to view them as places where they hurriedly attempt to find the information necessary to "fill in the blanks" in the laboratory report sheet that was to be handed in by a certain deadline later in the week. In the laboratories there seemed to be no opportunity to develop a sense of the "process of science." Questions did not have to be formulated nor was it necessary to design ways to answer questions that were being asked. No new information was being obtained by observation but the majority of the blanks could be filled in by finding the answers in textbooks. Many of the students were premeds already well into a competitive mode and there seemed to be no way for the students to develop an understanding of the role that collaborative efforts often contribute to scientific developments.

Can Introductory Labs be Real Science?

To what extent do the limitations of time and student numbers limit the inclusion of real scientific investigation into introductory laboratories? Is it possible to develop exercises that allow students to obtain real scientific data and to experience participation in the process of finding something that was previously unknown? I began to try to find ways in which students could do experiments that could be done collaboratively, would produce new and interesting data, were not overly expensive, and did not use materials or methods that could not be easily adapted to introductory labs, for example, methods using radioisotopes.

In considering this, I realized it would also be advantageous if similar methods could continue to produce new data that could be compared to the data produced by previous classes or other groups perhaps even in other schools. Another consideration is to what extent the exercise would provide opportunity to discuss basic biological principles or systems. Finally, the methods used would have to produce significant data within a few weeks or at least within the context of a single semester.

Cloning Genes from Bacteriophage Libraries

Having cloned genes from bacteriophage expression libraries, I realized that the methods used are relatively simple and not labor intensive and can, in many cases, produce interesting information. At one point, a postdoctoral student in my laboratory, had detected genes for protein kinases by screening phage expression libraries with an anti-phosphotyrosine monoclonal antibody (1-3). This procedure is based on the observation that there is normally no detectable phosphorylation of tyrosine in bacterial proteins but, when an active kinase is expressed in bacteria, the phosphorylated proteins can be detected by antibodies using an enzyme linked antibody assay system. *E. coli* bacteriophage expression libraries from a variety of sources can be used for the experiments. One of the advantages of the system is that any given system contains several kinases which may be detected with anti-phosphotyrosine antibodies so that the possibility of finding one or more kinase genes in any cDNA library selected is quite high. Phage plaques are produced and the proteins are transferred to nitrocellulose filters. The filters are reacted with an anti-phosphotyrosine monoclonal antibody and then with an enzyme linked anti-mouse antisera. Positive plaques are enriched, purified and the size of the inserted DNA determined. The isolated clones can be partially sequenced using standard primers and chemiluminescent sequencing or can be sent off to commercial laboratories to be sequenced. Sequences can be compared to each other and to sequences in existing databases.

Procedures are presented here for using these methods with groups of students in an introductory Biology course. They can be used over a three or four week period or continued over the course of a semester. Students work cooperatively in groups along with the instructor and the groups gather to discuss progress, compare sequences and then present their results at the end of the project. Potentially, groups from different schools could submit their sequences to a common database where they could be accessed and compared by the other groups. Students have opportunity to consider the various roles kinases play in cell

functions in a variety of organisms, to work with immunological and molecular genetic techniques and to analyze and compare original data.

Protein Kinases

Protein kinases play important roles in a wide variety of cellular functions including signal transduction and control of cell growth and division. There are a variety of web sources available for students to learn about the structures, functions, and activities of the various families of protein kinases starting with the Protein Kinase Resource at http://www.sdsc.edu/Kinases/pk_home.html.

Materials and Resources

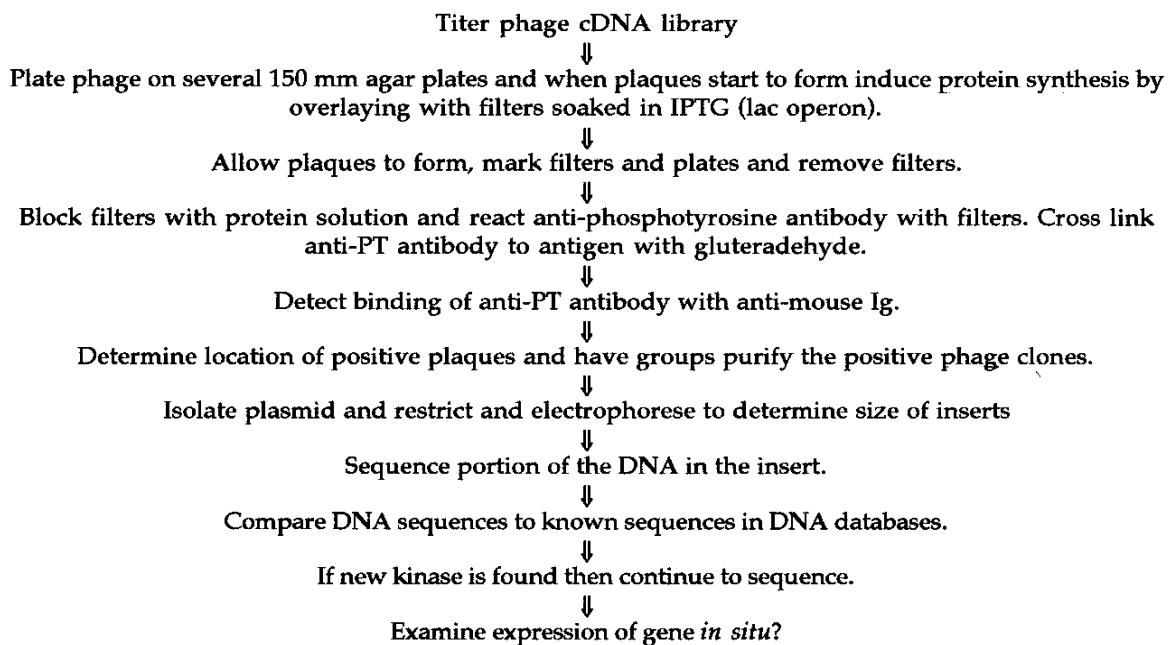
The materials and resources needed for the protein kinase gene exercise are listed here. Although in some cases specific suppliers are indicated, most are available from several suppliers. Many of the methods for bacterial and bacteriophage growth and maintenance are described in *Molecular Cloning: A Laboratory Manual* by Sambrook, Fritsch, and Maniatis (4).

1. Bacterial media, agar, plates and capacity to grow overnight cultures and incubate plates.
2. Anti-phosphotyrosine monoclonal antibody: This is obtained and used in the form of hybridoma culture supernatant. We use the monoclonal antibody IG2 described by Pasquale and Singer (3) and would be glad to provide information on obtaining and using the antibody. At least two other hybridoma lines (ATCC # 8190-HB and 1955-CRL) producing anti-phosphotyrosine antibodies can be obtained from the American Type Culture Collection. We are currently testing these for their ability to detect kinase genes in this assay.
3. Enzyme conjugated anti-mouse immunoglobulin reagent and reaction components. Peroxidase conjugated anti-mouse immunoglobulin (heavy and light chain) produced by Cappel Labs can be obtained from ICN (<http://www.icnpharm.com>). Their reagent can usually be diluted at least 1/500 for use in these experiments. The enzyme substrate and reaction indicator H₂O₂ and 4-Chloro-1-Naphtol can be obtained from Sigma Chemical Corp.
4. cDNA expression libraries in bacteriophage: Several suppliers such as Stratagene and CloneTech sell libraries from a wide variety of organisms and cell types. Also, since only a few ul of a library usually contain sufficient phage for a screening experiment, investigators using such libraries may be willing to donate a sample for a laboratory exercise particularly with the possibility that they may obtain clones of genes for kinases that were not previously identified.
5. Host strains of *E. coli*: Several appropriate strains are available from suppliers. We use *E. coli* strain Y1090r obtained from Stratagene.
6. IPTG: Isopropyl-Thio-Galactoside, used to induce the expression of the proteins in the bacteria infected with phage, can be obtained from various suppliers.
7. Restriction endonucleases. From various suppliers. Useful if you would like to characterize the size of the insert in the kinase gene clone detected. The particular enzyme would depend on the site into which the cloned sequences were inserted when the library was produced.

8. **DNA sequencing:** Departments with equipment and the capacity to have students do DNA sequencing can order the appropriate primers and do the initial sequencing on the clones isolated. In the past few years DNA sequencing facilities utilizing automated equipment have been developed to the point where it is quite easy to find a facility that will provide initial sequence data on an isolated clone for a minimal expense. Clones isolated by the class can be sent off for sequencing and the data provided used to compare the clone to sequences for previously identified kinase genes.
9. **Access to DNA databases:** sequence databases that provide search functions are available on the internet and can easily be utilized to search for sequences related to the sequence of the kinase gene(s) isolated by the classes. The website of the National Center for Biotechnology Information provides connections to such databases as well as other resources of interest to students (<http://www.ncbi.nlm.nih.gov/>).

Protocol Flowchart

The chart below briefly outlines the protocol used for screening for kinase genes. This includes full sequence of experiments that may lead even to study of the *in situ* expression of the gene in the organism from which the gene is derived.



Method for Phage Plaque Screening

Prior to the experiment the phage library should be titered so that the amount of phage suspension necessary to produce about 5×10^4 plaques is determined. This amount should be added to each tube of recipient that is plated on a 150 mm plate. This allows screening of the maximal number of plaques without complete lysis of

the bacteria lawn. If 10 - 20 of these plates are screened at one time at least one kinase gene should be isolated. In most cases several will be detected. In our initial screening of a human brain cDNA expression library we isolated five different kinase clones.

Production of Plaques and Transfer of Proteins to Filters

1. The recipient strain of *E. coli* is streaked out on an NZY agar plate with the appropriate antibiotic(s). Y1090r- is streaked on NZY plus XXX ampicillin.
2. Take a colony from the recipient plate and start an overnight culture of the recipient in TB broth + 10 mM MgSO₄ + 0.2% maltose.
3. Before the plating procedure is started the sterilized top agar can be remelted in a microwave and placed in a waterbath to equilibrate to 48°C. The plating process is easier if the plates are prewarmed to 37°C for about an hour before use.
4. Read OD₆₀₀ in a Spectronic 20 (overnight culture of Y1090r- usually has OD₆₀₀ of ~ 1.2).
5. Centrifuge cells 10 minutes at 2000 rpm.
6. Decant the supernatant, suspend the pellet and add 10 mM MgSO₄ to a final OD₆₀₀ of 0.5.
7. Mix aliquots of phage to 600 ul cells in disposable 10 - 15 ml tubes (200 ul for 100 mm plates).
8. Incubate cells and phage at 37°C for 15 minutes.
9. Add 6.5 ml of top agar (3 ml for 100 mm plates) to each tube of infected bacteria and spread evenly over an NZY plate.
10. Incubate the plaques at 42°C until small plaques form (~ 3.5 hours).
11. Treat nitrocellulose filter (sterilized by autoclaving) with IPTG (10 mM): 30 minutes prior to use, wet nitrocellulose filters by submerging them in the IPTG solution until completely wet. Place them slowly into the solution starting at the edge and allowing the solution to be drawn into the filters by capillary action. Put onto blotting paper to air dry.
12. Number each of the filters and mark each with three marks asymmetrically distributed. Marks corresponding to these marks will be made on the plates after they are put onto the plates. This allows picking of positive plaques from the plates by allowing positive spot of enzyme reaction detected on the filters to be correlated with a position on the agar plates.

13. Place filters on plates, mark the plates with lines corresponding to the marks on the filters, number the plates with the filter number, and incubate them at 37°C for 3.5 hours. At this point the plates with filters can be placed in a cold room or refrigerator overnight.

Washing and blocking of filters

During the washing steps rocking the container gently using a shaker or rocking platform facilitates the process.

14. Carefully remove filters with forceps and wash them in TBST (If the top agar sticks to the filter try cooling the plate to 4°C for 4 hours or more.)
15. Immerse the filters in TBST and remove any remaining top agar with a gloved hand or smooth metal rod.
16. Combine the filters in plastic container or heat seal bag and wash 3-5 times with 10 ml per filter of TBST for at least 15 minutes.
17. Immerse filter in blocking solution (5% (wt/v) dry milk in TBST) and agitate gently for 1 hour at room temperature in order to block remaining protein binding sites. (Filters can be stored in blocking solution with 0.02% sodium azide (wt/v) at 4°C in a sealed bag.)

Incubation with anti-phosphotyrosine antibodies

18. Transfer filters into 10 ml per filter of primary antibody solution. This can be hybridoma supernatant or dilution of antibody in blocking solution. Incubate with gentle shaking for at least 1 hour at room temperature.

Fixation of first antibody with gluteraldehyde

Fixation of the primary antibody assures that this antibody is not removed during subsequent steps. Phosphate buffered saline (PBS) rather than Tris buffered saline (TBS) is used in this part of the procedure because the Tris interferes with the reaction of the gluteraldehyde with proteins.

19. Wash 3X in 0.05% Tween 20 (v/v) in PBS ~ 15 minutes each wash.
20. Fix with 0.1% gluteraldehyde in cold PBS for 15 minutes.
21. Wash 1X in PBS for 15 minutes.
22. Block with 5% dry milk in PBS for 20 minutes
23. Wash 2X in 0.05% Tween 20 (v/v) in PBS ~ 15 minutes each wash

Reaction with Peroxidase-Conjugated Antibody

24. Transfer filter into 10 ml per filter of fresh blocking solution containing peroxidase conjugated anti-mouse Ig (1/500) and incubate with gently agitation for 1 hour at room temperature.
25. Wash filters 3 - 5 time in 10 ml per filter TBST for 5 minutes each time to remove any residual unbound or non-specifically bound conjugate.
26. Remove residual Tween-20 by doing a final wash in 10 ml per filter TBS alone.

Color development on filter

27. Prepare 90 mg 4-Chloro-1-Naphtol in 60 ml methanol and add to 300 ml TBS along with 120 ul H_2O_2
28. Filters are immersed in this substrate mixture and incubated at room temperature with gentle shaking. Staining of positive plaques can usually be detected within 15 minutes.
29. Stop reaction with by washing with H_2O .

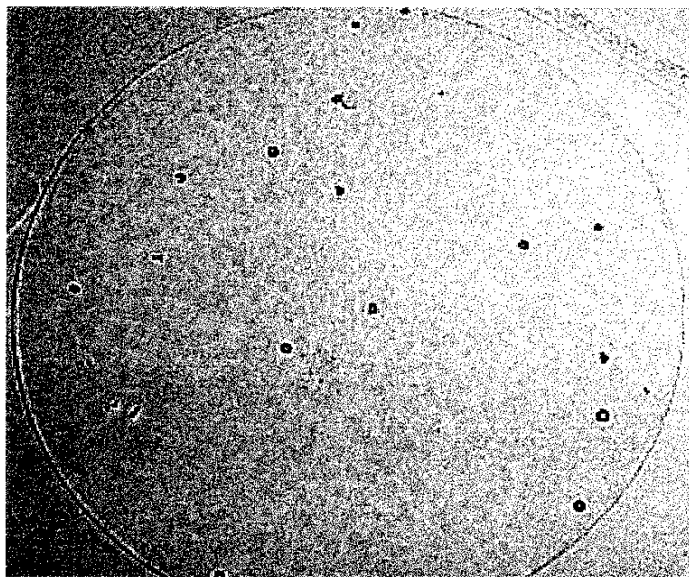


Figure 1. A mixture of positive and negative plaques on a nitrocellulose filter. The plaques were made using a semipurified population derived by picking a positive plaque on the basis of an initial screening. This represents a secondary screening done using a 100 mm plate. A plaque corresponding to a positive (darker) plaque will be picked from the corresponding plate and the phage picked will again be screened in order to purify the positive phage clone.

Purification of Phage from Positive Plaques

On the basis of the position of positive spots on the filters, a plug of agar is removed from the corresponding location on the plates. Plugs can be removed from the agar by using the large end of a Pasteur pipette. The agar plugs are suspended overnight in SM buffer to allow the phage to diffuse into the buffer. Dilutions of this phage suspension are used to make plaques on 100 mm plates. The previous screening steps are followed and a mixture of positive and negative colonies will be detected. This procedure of enrichment is repeated 2 - 3 times until a purified positive phage clone is isolated.

DNA Sequencing

DNA from the purified phage clone can be sent to a DNA sequencing facility for sequencing. Data returned, usually in the form of a computer data file, can be used as a basis for comparing the sequence to that of other kinases. The students have the satisfaction of being able to produce original and significant data as well as realizing that such data has significance in the fuller context of research done by other investigators. They also see the importance of cooperation with other scientists such as those in the sequencing facility and those that establish and maintain the DNA databases.

Discussion

In the context of the isolation of the kinase gene sequences there is opportunity to discuss a variety of biological phenomena and principles. Besides discussing the experimental design and the need for controls there is opportunity to discuss, for example (1) the functions of kinases, (2) post-translational modification of proteins, (3) signal transduction, (4) viral and phage life cycles, (5) phage receptors induced by the maltose included in the growth media, (6) gene expression and induction of the lac operon, (7) recombinant DNA techniques, (8) plasmid structure, function and purification, (9) antibody specificity, (10) enzyme reactions, (10) DNA sequencing and sequence comparisons including the use of computers and databases in molecular biology.

We have currently been using these techniques with a small group of students and plan to begin use of the exercise in our introductory labs during the next school year. We would be interested in cooperating with other schools interested in comparing results and sharing DNA sequences and eventually setting up a database of sequences kinase genes from various tissues and organisms.

Literature Cited:

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4. Sambrook, J., Fritsch, E.F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual, Second Edition*. Cold Spring Harbor Laboratory Press, CSH, New York, 1989

Appendix

Recipes for buffers and reagents used in the outlined procedures:

TBS

Tris buffered saline
20 mM Tris-HCl pH 7.5
150 mM NaCl

TBST

Tris buffered saline and 0.05% Tween-20
TBS + 0.05% Tween-20 (v/v)

SM buffer

100 mM NaCl
50 mM Tris pH 7.5
10 mM MgSO₄

NZY broth

Per Liter:

5g NaCl
2g MgSO₄ 7H₂O
5g Yeast Extract
10g NZ Amine (casein hydrolysate)

NZY plates

Add 15 g Difco Agar/liter NZY broth
Autoclave and pour
Allow ~ 80 ml/150 mm plate