

## Isolation of Novel Genes from a cDNA Library

### INTRODUCTION

In this laboratory activity, you will utilize techniques used by genetic researchers to discover previously unidentified genes from eukaryotic organisms. The lab may be conducted as a demonstration (i.e., screening for known genes) or as a novel experiment (screening for unknown genes). The kind of library you will work with (i.e., human, *Arabidopsis*, *Ceratopteris*, etc.) and the gene you attempt to identify will be determined by your instructor. If your class is able to conduct a screen for previously unknown genes, this experiment could lead to additional studies (a student and/or faculty research project) and the results could ultimately be published! Your goal for now will be to use an antibody-based selection scheme to identify bacterial hosts infected with viral vectors carrying a specific gene.

The library you will use was constructed by inserting cDNA copies of mRNAs from a eukaryotic organism into bacteriophage vectors (also simply called *phage vectors*). *Bacteriophages* are viruses that specifically infect bacteria, and *vectors* are entities that carry and replicate foreign DNA. The phage vectors in this library have been engineered in such a way that the foreign genes will be expressed (i.e., both transcribed and translated). To identify and isolate particular genes in this library, you will use a selection scheme that employs antibodies against these proteins (described in detail later). The cDNA library contains copies of many genes (besides the ones you are trying to identify), but because of the specificity of the detection assay (antibody-based), only bacteria with the virus containing the genes of interest will be detected.

It will require several days to conduct all aspects of this experiment (described later under “Procedures”). The class will divide the responsibility for conducting different parts between various groups of students. Your instructor may choose to have some steps of the experiment done by a lab assistant, rather than assigning these responsibilities to student groups. You should read the following description of the selection scheme and familiarize yourself with all of the steps before coming to lab. Your instructor will then explain how your class will conduct the specific steps of the experiment.

*Note:* A good deal of reading should be done prior to coming to lab. The amount of reading is more than usual, but it is *essential* to your understanding that you read the following to prepare for lab. Otherwise, this will seem like a “cookbook” exercise.

### THE SELECTION SCHEME

Eukaryotic organisms possess tens of thousands of genes, and a given cDNA library may contain copies of most or at least a large percentage of these genes. Once a cDNA library has been constructed for a given organism, the next challenge is finding genes of interest. There are several ways of doing this, but all of them involve growing many, many random representative vectors from the library and

somehow screening them all to isolate a small number that carries the gene of interest. A screening strategy often described in introductory textbooks involves finding the gene of interest by “probing” the library with a labeled polynucleotide. This approach has been used quite successfully, but it has certain limitations, including the fact that some sequence information for the gene of interest must be available (to design the polynucleotide probe). You will be using a different approach that employs antibodies against the protein made from the gene of interest as the probe. This eliminates the requirement for specific sequence information prior to screening.

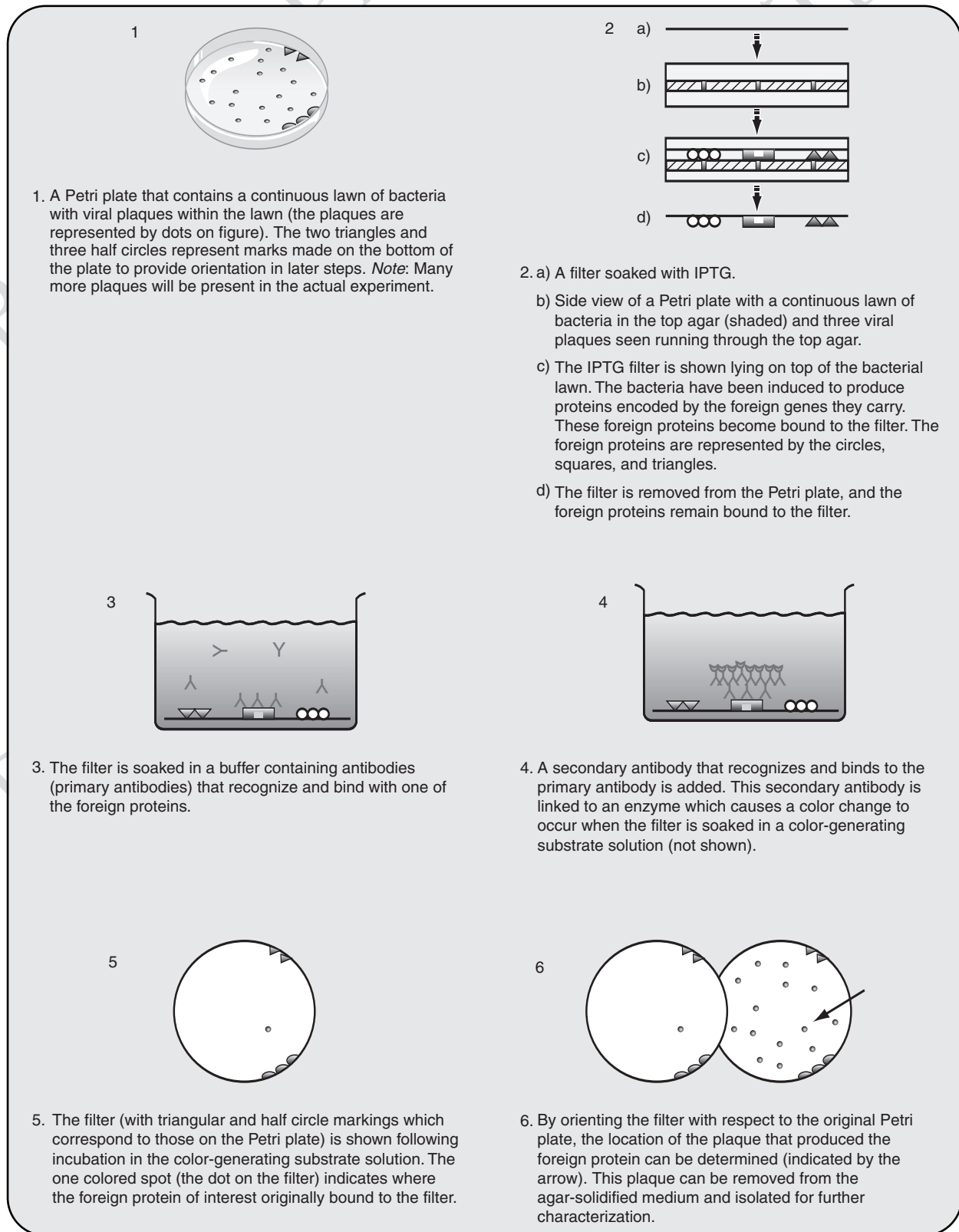
**Q1** Assume that you want to screen a cDNA library for a gene that has not yet been sequenced. How would the antibody for the protein from this gene be generated? (*Note: You could provide a highly detailed answer to this question; however, an answer of one to several sentences will be sufficient.*)

The multistep procedure for antibody-based screening (see Figure 12.1) is described in detail later. Briefly, it involves the following elements: (1) growing the phage-infected bacteria in a layer of “top agar” (described below) on nutrient agar plates, (2) stimulating gene expression with isopropyl  $\beta$ -D-thiogalactoside (IPTG)-soaked filters and allowing expressed proteins to adhere to the filters, (3) incubating the filters with the primary antibody (which recognizes the protein of interest), (4) incubating the filters with a secondary antibody (which recognizes the primary antibody and carries an enzyme “label”), (5) “developing” the filters (the enzyme linked to the secondary antibody causes a color change), and (6) identifying the location of the gene of interest on the culture plates and removing the bacteriophages from the culture at that location.

Note that this detection method can only be used successfully for genes that are present in eukaryotes but not in bacteria. Otherwise, the antibody would cross-react with bacterial gene products.

### Growing Phage-Infected Bacteria

In this part of the protocol (Steps 1 to 7 in the Procedures), bacteria and phage vectors are combined in predetermined quantities such that hundreds of thousands of infections can take place. This is necessary to increase the likelihood of finding the gene of interest. The phage-infected bacteria are grown in such a way that the culture plates are covered with a “lawn” of bacteria (growing in an upper layer known as *top agar*) with infected cells scattered throughout the lawn. As the cultures are incubated, the infections spread, forming clones of infected bacteria that are visualized as “plaques” on the bacterial lawn. A plaque is a clear spot in the top agar where all of the bacteria have been lysed. Your instructor will determine the appropriate concentration of bacteria and phages for your experiment. It is ideal to have so many plaques on a culture plate that they are densely packed together, yet few enough that it is still possible to identify individual plaques.



**Figure 12.1** – An overview of the multistep process for antibody-based screening of a cDNA library in a phage expression vector. See the text and figure notes for details.

### Overlaying Cultures with IPTG-Soaked Filters

The phage expression vectors you will be using can be stimulated with the chemical IPTG to induce expression of their foreign genes (Steps 8 to 11 in the Procedures). They have been engineered so that a genetic element that functions as an “on/off” switch has been placed into the vector DNA just prior to where the foreign DNA is inserted. This on/off switch is the well-known *lac operon* that some bacteria use to naturally regulate the production of lactose-metabolizing enzymes. If you have never studied the *lac operon* before, or you have, but have forgotten what you learned about it, you should take time now to review this classic example of genetic regulation. Most up-to-date general biology textbooks describe this system, as do virtually all genetics textbooks.

**Q2** Now that you have reviewed the *lac operon*, briefly answer the following questions related to its function:

A. What is the “inducer” in the *lac operon* system?

B. What is the “repressor” in the *lac operon* system?

C. How do the inducer and the repressor interact with a site called the “operator” to induce gene expression?

The chemical IPTG is a synthetic compound that takes the place of lactose for the purpose of this experiment. Unlike lactose, it is not metabolized by the structural genes of the lac operon; however, it is still able to “turn on” gene expression. After the phage-infected bacteria have been growing for several hours, filters soaked with IPTG are laid on top of the culture medium. The filters and culture plates are marked so that after the filters are removed, they can later be realigned with the culture plate (described later). The IPTG-soaked filters are made of a material that has a high affinity for proteins. Thus, when the IPTG stimulates the expression of the foreign genes, the gene products (proteins) become adhered to the filter. At this point, it becomes possible to manipulate the filters to screen for evidence of the gene of interest, while allowing the bacteria that are infected with phage vectors to remain on the culture plates. After the location of the gene of interest is identified on the filter, the filter can be realigned with the culture plate to determine where the “positive” plaque is located.

The steps described so far can be accomplished in one day. The remainder of the experiment can be accomplished on the following day or over a period of days thereafter.

### **Incubating Filters with the Primary Antibody**

This part of the experiment (Steps 12 to 19 in the procedures) involves washing and blocking the filters (discussed later) and allowing them to react with the primary antibody. It is the reaction with the primary antibody that provides the specificity in this experiment so that only certain genes are identified by the screen. Any of a very large number of possible antibodies could be used during this step; however, you should follow two main guidelines to select an antibody that is most likely to provide positive results. First, the antibody should be one that reacts only with proteins produced by eukaryotic organisms. Otherwise, if bacteria also produce this protein, all of the bacteria growing on plates in this experiment will produce a positive signal. Second, the antibody should have been made from a protein that should be similar to a protein in the organism from which your library was made.

There are several ways that this criterion can be met. For example, the antibody could be specific for a protein (or a region of a protein) that appears to be highly conserved. In such cases, it is possible that an antibody made for an organism in one kingdom (e.g., humans), could even react with proteins from an organism in another kingdom (e.g., a fern). Or, the antibody could be specific for a protein from the actual species for which your library was made or for a closely related species.

Another consideration in selecting the antibody is related to the likely abundance of the mRNA for that protein in your organism. Since the cDNA library was made from mRNA, the number of copies of a given type of cDNA present in your library will be based on the original level of that mRNA in your organism. For example, the number of copies of tubulin cDNAs (a high-abundance “housekeeping” gene) in a given library is likely to be much higher than the number of tyrosine kinase cDNAs (tyrosine kinases are regulatory genes). This means that you are more likely to have success if you choose to search for a gene that is highly transcribed. However, on the other hand, the less commonly transcribed genes are often much more interesting!

It is also possible to conduct this experiment with built-in controls, so that obtaining some positive plaques is almost guaranteed. For example, you could screen one library for a previously unidentified gene, but include some isolated phages (on a control culture) from another library that are known to carry that gene. If such a control sample of phages is available, this step is recommended.

Many other steps conducted before and after addition of the primary antibody are either “washing” or “blocking” steps. Both of these types of procedures help to reduce the amount of background signal (i.e., they help eliminate false positives). The washing steps are necessary to remove material that has become loosely adhered to the filter in a nonspecific way. The blocking step uses milk proteins to “cover” unbound areas of the filter (places where no foreign gene has adhered). This step is

done just prior to adding the primary antibody so that the antibodies will not adhere to the empty spaces on the filter in a nonspecific way.

A step in this part of the experiment also involves gluteraldehyde fixation. During this step, the gluteraldehyde interacts with the protein and the antibody to create covalent bonds between them. This ensures that the antibody will remain bound to the filter during the remaining steps.

### **Incubating Filters with the Secondary Antibody**

Steps 20 to 22 in the Procedures

The secondary antibody is “tagged” with an enzyme (a peroxidase) that causes a color reaction to occur (see the next section) on the filter where protein from positive plaques is located. The secondary antibody (usually an antibody made in a goat) recognizes the primary antibody (usually an antibody made in a mouse) and binds specifically to it. Without adding the secondary antibody, the primary antibody cannot be detected (no color reaction will occur).

There are also washing and blocking steps associated with the incubation with the secondary antibody. These steps serve essentially the same purpose as the previous washing and blocking steps associated with the primary antibody.

### **Developing the Filters**

Steps 23 to 25 in the Procedures

During this stage of the experiment, the filters are exposed to a solution that contains a colorless substrate for the peroxidase enzyme on the secondary antibody. As the enzyme interacts with this substrate, it transforms it into a bluish/purple-colored product. This product becomes localized on the filter.

### **Locating the Gene of Interest and Isolating Positive Plaques**

Steps 26 to 29 in the Procedures

After the location of the foreign protein for the gene of interest has been identified on the filter, it is necessary to use the filter to locate the positive plaque on the experimental culture plate(s). This is accomplished by using filters and culture plates marked with corresponding unique indicators (i.e., nicks or holes in the filters and corresponding permanent pen markings on the bottoms of the culture plates). After positive spots have developed on one or more of the filters, plastic sheets (i.e., overhead transparencies) are used to make “maps” of the filters, showing where the positive spots occurred.

These are then placed beneath the culture plates and used as guides for removing “plugs” from the culture medium that contains the positive plaques. The plugs are removed using the wide ends of glass pipettes and then transferred into a phage elution buffer. The plugs will also probably contain other plaques (which lack the correct foreign gene), and further “subcloning” (repeating this procedure with material from these plugs) will be necessary to purify the sample. However, this lab activity will be complete after the plugs containing positive plaques have been removed.

**Q3** Now that you have read the description of this experiment, explain in your own words why two antibodies are used in this experiment.

- A. What is the specific role of each of the two antibodies?
- B. Can you think of any advantages to using two antibodies and having the second one be “enzyme linked,” rather than using an enzyme-linked primary antibody? Explain. (*Hint: As you try to answer part B of this question, it may be helpful to consider the implications of part 4 of Figure 12.1.*)

## PROCEDURES

It will require several days to conduct all aspects of this experiment as described here. The class will divide the responsibility for conducting parts of the experiment among various groups of students. Your instructor may choose to have some steps done by a lab assistant, rather than assigning these responsibilities to student groups. *Before beginning, be certain that you know which parts of this experiment you are responsible to conduct!*

### Specific Steps for Phage Plaque Screening:

Steps 1 to 7: Growing Phage-Infected Bacteria

**Step 1:** Grow an overnight culture of the bacteria used for screening the library. On the following day, read the OD<sub>600</sub> of this culture in a spectrophotometer to estimate the initial density.

**Step 2:** Centrifuge the overnight culture 10 minutes at 2,000 rpm.

**Step 3:** Decant the supernatant, and suspend the pellet in a 10 mM MgSO<sub>4</sub> solution to a final OD<sub>600</sub> of 0.5 (this value may be changed for some strains).

**Step 4:** Mix aliquots of the phage library to 600 µl of cells in disposable 10- to 15-ml tubes for screens conducted on 150-mm culture plates (200 µl for 100-mm plates). *Note:* Your instructor will determine how many microliters of the phage library to add.

**Step 5:** Incubate the cells and phages at 37°C for 15 minutes.

**Step 6:** Add 6.5 ml of warm top agar (3 ml for 100-mm plates) to each tube of infected bacteria, and spread evenly over a plate containing NZY medium that was kept in a 37°C chamber just before use.

**Step 7:** Incubate the plaques at 42°C until small plaques form (ca. 3.5 hours).

### Steps 8 to 11: Overlaying Cultures with IPTG-Soaked Filters

**Step 8:** Make asymmetrical, unique nicks (e.g., cut triangles and/or circles) on edges of the filters and autoclave them. After removing the filters from the autoclave, attempt to keep them as sterile as possible before placing them onto the cultures (use sterile IPTG, handle with sterile forceps, etc.).

**Step 9:** Treat the filters with 10 mM IPTG solution at least 30 minutes prior to use, and wet the filters by submerging them in the IPTG solution until they are completely wet. Place them slowly into the solution, starting at the edge, and allow the solution to be drawn into the filters by capillary action. Put the filters onto blotting paper briefly to dry them somewhat (the filters should remain moist, but not be sopping wet).

**Step 10:** Place filters on the surface of the culture plates, and mark the bottoms of the plates with lines corresponding to the marks on the filters. These corresponding marks should be made as carefully as possible so that any positive plaques can be easily isolated at the end of the experiment.

**Step 11:** Incubate the plates at 37°C for 3.5 hours. After this, the plates with filters can be placed in a cold room or refrigerator overnight.

### Steps 12 to 19: Incubating Filters with the Primary Antibody

During many of the remaining steps, the filters will be soaked in various solutions. The volume of each solution should be about 10 ml per filter. While the filters are soaking in solutions, they should be rocked gently, using a shaker or rocking platform.

**Step 12:** Carefully remove filters with forceps, and wash them in TBST solution. Immerse the filters in TBST and remove any remaining top agar with a gloved hand (wash gloves first) or smooth, metal rod. Combine the filters in a plastic container or a heat-sealed bag, and wash 3 to 5 times with TBST for at least 15 minutes each.

**Step 13:** Immerse the filters in blocking solution (5% weight/volume dry milk in TBST) and agitate gently for 1 hour at room temperature to block remaining protein binding sites. (Filters can be stored for at least several days at this point in blocking solution with 0.02% sodium azide weight/volume at 4°C in a sealed bag. *Sodium azide is poisonous; handle with care!*)

**Step 14:** Transfer the filters into a primary antibody solution. Incubate with gentle shaking for at least 1 hour at room temperature. Turn (i.e., “flip”) and spread the filters periodically.

**Step 15:** Wash three times in 0.05% Tween 20 (v/v) in PBS for at least 15 minutes each wash. 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 mediated by the glutaraldehyde in the next step.

**Step 16:** Fix the primary antibodies to the proteins by washing with 0.1% glutaraldehyde in cold PBS for 15 minutes. *Handle the glutaraldehyde with care — it is toxic!*

**Step 17:** Wash once in PBS for 15 minutes.

**Step 18:** Block with 5% dry milk in PBS for 20 minutes.

**Step 19:** Wash twice in 0.05% Tween 20 (v/v) in PBS for at least 15 minutes each wash.

### Steps 20 to 22: Incubating Filters with the Secondary Antibody

**Step 20:** Transfer the filters into 10 ml per filter of fresh TBST blocking solution containing peroxidase conjugated antimouse antibody, and incubate with gentle agitation for 1 hour at room temperature.

**Step 21:** Wash the filters 3 to 5 times in 10 ml per filter TBST for 5 minutes each time to remove any residual unbound or nonspecifically bound enzyme-conjugated antibody.

**Step 22:** Remove residual Tween 20 by doing a final wash in 10 ml per filter TBS alone.

### Steps 23 to 25: Developing the Filters

**Step 23:** Prepare 90 mg 4-chloro-1-naphthol in 60 ml methanol, and add this to 300 ml TBS along with 120  $\mu$ l of  $H_2O_2$  (this volume is sufficient for up to about 20 filters).

**Step 24:** 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 to 25 minutes. Proteins from different plaques will stain with different intensity (depending on the specific structure of the protein and antibody binding). The vast majority of plaques should not produce a positive reaction. True positive spots will appear uniquely dark compared to most neighboring spots (however, slight background staining is likely).

**Step 25:** Stop reaction by washing with water.

### Steps 26 to 29: Locating the Gene of Interest and Isolating Positive Plaques

**Step 26:** Dry the filter(s) that indicate the presence of positive plaques, and use a plastic sheet (i.e., an overhead transparency) to draw the outline of the filter (including the unique nicks on the edges) and the location of the positive plaque(s). Do this very carefully so that you will be more likely to actually isolate the positive plaques in the next step.

**Step 27:** Position the culture plates on the plastic sheets that show the locations of positive spots, so that the marks on the plates and marks on the plastic sheets are aligned.

**Step 28:** Using the wide end of a sterilized glass pipette, and, maintaining sterility as much as possible, remove a plug of the culture medium that should contain the positive plaque (based on the mark on the plastic sheet beneath the plate).

**Step 29:** Transfer this plug into 1 ml of sterile SM buffer containing 2 drops of chloroform.

The phages present in this plug of culture medium will diffuse out into the SM buffer after about 24 hours. Although the plug you isolated probably contains several phages carrying different foreign genes, the gene of interest should be present at a relatively high titer. It will now be possible for someone eventually to isolate only the phage carrying the gene of interest by repeating the prior protocol using various dilutions of the phage you isolated. The ultimate goal will be to sequence this newly discovered gene and so contribute to our existing knowledge about the structure and variation of eukaryotic genes.

- Q4** For this question, your instructor will ask you to respond for a particular organism of his or her choice. Assume, that you have a cDNA library for the organism your instructor has indicated. If you could do this experiment again using this cDNA library and any antibody of your choice, what gene would you try to identify and why? In your answer, include technical reasons that you would choose a particular gene (how likely is it to work?), and also describe what scientific value might be associated with isolating this gene (i.e., how could your discovery be used in the future?). You should try to identify a gene that has not already been found and sequenced. To help you identify such a gene, go to the National Center for Biotechnology Web page for Nucleotide searches ([www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide](http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide)), and verify that the gene you're interested in hasn't already been found.

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