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Molecular Biology in Real Time: Tricks and Tips for Turbo-charging and Student-proofing Standard Protocols

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At the University of Maryland we have made a concerted effort to develop and incorporate "cutting-edge" scientific experiences for our undergraduates at all levels. Students in our introductory molecular and cellular biology course for science majors are given the opportunity to work with technologies that are central to modern research labs. During two three-week lab modules students get hands-on experience with polyacrylamide gel electrophoresis of proteins (PAGE), bacterial transformation, plasmid mini-preps, PCR, and DNA electrophoresis. Traditional protocols for some of these procedures take to much time to accomplish in a single lab period, use hazardous chemicals, and/or are to complicated/delicate for novices to complete successfully. We will discuss ways to make PCR, DNA minipreps, and gel electrophoresis faster, simpler, and safer for students. Using these methods it is possible to perform colony PCR, complete a plasmid miniprep, and look at the results on an agarose gel all within a single 3 hour lab period, without ethidium bromide or UV light.

Keywords: electrophoresis, plasmid miniprep, PCR

Introduction

There is a growing interest in adopting molecular biology methods for undergraduate teaching labs. Unfortunately, the protocols for applying technologies such as PCR or electrophoresis are often long and involved, or use hazardous materials that are not suitable for use by novice lab students. In this workshop a set of procedures were presented that make three core procedures faster and safer and thus more suitable to the teaching lab (PCR, plasmid miniprep, and agarose electrophoresis). The principals behind developing these modified protocols are general, and instructors are encouraged to apply them to other methods:

1. Research methods need to be perfect; teaching methods can be "good enough"
2. Deconstruct methods; find and eliminate steps that are not critical
3. Understand how technologies work and experiment; if you have an idea, try it!

Student Outline

Background

A common source of food poisoning is ingestion of *E. coli* from contaminated raw foods. *E. coli* is a normal part of the intestinal microbial ecosystem but can be harmful to other parts of the digestive tract. These bacteria belong to a group called the *Enterobacteriaceae* that also includes less benign members such as *Salmonella*. A common clinical test to identify E. coli is the ability to metabolize the simple sugar lactose (lac+), but rare strains of *Salmonella* have evolved that are also lac+ and lead to misdiagnosis of cases of food-poisoning. Molecular biology methods have proven useful in designing more specific tests for identifying clinically relevant bacterial strains.

The most powerful tool for this purpose has been **PCR**, the polymerase chain reaction, which allows selective amplification of the DNA for "markers" based on unique genes or sequence polymorphisms. PCR can multiply an individual gene into millions of copies very rapidly and provide enough DNA to easily analyze using **agarose gel electrophoresis**.

Additional tools such as the **DNA miniprep** allow the separate analysis of bacterial genomic DNA and plasmid DNA, which often is the source of rapidly evolving antibiotic resistance. Genomic DNA in bacteria typically consists of a single large, circular molecule that includes all of the required genes for the organism to function. Plasmids are small circular DNA molecules that are distinct from and supplemental to the genomic DNA - they are not required but can provide additional material to improve the survival of the host bacteria.

What is PCR?

The polymerase chain reaction, or PCR, uses the components of the native cellular DNA replication machinery to make copies of, or **amplify**, specific pieces of DNA. In a cell, the DNA needs to be replicated, or copied, prior to each division. This is accomplished by the enzyme **DNA polymerase** that is recruited to short, complementary **primers** that bind to specific locations along the original DNA **template**. PCR uses a special polymerase enzyme (*Taq*) originally isolated from a heat-loving microbe. Unlike many enzymes, *Taq* can be heated repeatedly without denaturing, and is the basis for a technique call thermocycling. A mixture of template DNA, primers, nucleotides, and *Taq* is repeatedly heated and cooled so that new DNA copies are produced each cycle, amplifying the sequence of interest.

How does a PCR test work?

A *PCR test* is any assay that uses PCR to analyze a piece of DNA known to differ between genomes. A particular assay may test for presence/absence of a gene that is known to occur in one genome but not another, or it may be based on a **polymorphism** in a gene between different strains or associated with a disease. A polymorphism in the DNA sequence could make a primer no longer work so you either get amplification or you don't, or it may produce products that differ in length. Either result can be easily examined using gel electrophoresis.

This exercise will test for the presence/absence of three markers using a technique called **multiplex PCR**. The first marker is common to all *Enterobacteriaceae*, while the other two are unique to *Salmonella*.

What is a plasmid miniprep?

Much of molecular biology is based a technique called **cloning** that involves isolating a gene or other piece of DNA, putting it into a plasmid, and using bacteria as little factories to make precise copies of the plasmid. If you want more plasmid, you simply grow more bacteria! To recover clean plasmid, getting rid of everything else, the miniprep is typically used. This technique involves four steps: (1) lysis of bacterial cells, (2) precipitation of DNA from the solution, (3) washing away lipids, proteins, RNA, and genomic DNA, and (4) resuspending the plasmid DNA in a small volume.

How does DNA electrophoresis work?

Gel electrophoresis is used to separate DNA molecules by size, based on the fact that DNA has a negatively charged backbone and will move in an electric current. For DNA, a seaweed product called **agarose** is often used as it forms a matrix suitable for separating a wide range of molecules. The agarose retards the movement of molecules in proportion to their size so small molecules move faster than large molecules when an electric current is applied. A DNA specific dye is added to the gel to make the different **bands** of DNA visible, and a **ladder** of known-size DNA fragments is used to judge the size of DNA molecules observed.

Procedure I: Colony PCR

There are many methods to isolate and purify DNA from different cell types. Some *downstream* applications, such as restriction digests and gene cloning, require very clean DNA without contamination by lipids, polysaccharides, proteins, or RNA. Simple PCR for gel electrophoresis, on the other hand, is possible without purification as long as the target DNA is in solution and there is nothing present that will interfere with the DNA polymerase enzyme. Colony PCR is a simple method that involves lysis of bacterial cells in water, which also dilutes cell debris and contaminants, followed directly by PCR and agarose gel electrophoresis.

1. Pipet 50 µL distilled water into a clean 1.5 ml microcentrifuge tube.
2. Open a bacterial growth plates and choose a colony; use a sterile toothpick to gently lift the colony off the agar plate. Be careful not to scrape the agar!
4. Dip the tip of the toothpick with the colony in the water in your microcentrifuge tube and stir briskly for a few seconds. This will pop the cells giving you bacterial lysate.
5. Close the tube and discard toothpick properly.
6. Take your tube to the front of the room, and pipet 8 µl of your bacterial lysate into one tube in a strip as directed by your TA.
7. Your TA will add 12 µl of PCR Master Mix to each tube and seal the caps tightly. The strip of tubes will then be placed in the PCR machine (thermocycler) and run with the following program:

Step 1. Enzyme activation:	_____° C for _____ minutes
Step 2. Amplification:	(_____ cycles)
Denaturation	_____° C for _____ seconds
Annealing	_____° C for _____ seconds
Extension	72° C for _____ seconds
Step 3. Final Extension:	72° C for 3 minutes

8. When the program is complete your TA will remove the tube strip from the PCR machine, cut off your tube, and give it to your group.
9. Add 4 µl of DNA Loading Dye to your tube and pipet up and down to thoroughly mix with your PCR product.
10. Analyze reaction products by agarose gel electrophoresis.

Procedure II: Plasmid Miniprep

In this exercise you will isolate plasmid DNA from a culture of the bacterial strain. Culture media with ampicillin added was inoculated and grown overnight, resulting in a dense population of antibiotic resistant bacteria. You will use a plasmid DNA miniprep procedure to determine if the ampicillin resistant bacteria are carrying plasmid.

1. Place (a) two clean microcentrifuge tubes and (b) a miniprep column with collection tube in a rack.
2. Get one microcentrifuge tube and pipet 600 µl of bacterial culture provided.
3. Add 100 µl of 7X Lysis Buffer (blue) to your tube, and immediately cap tube and turn upside down several times to mix. If all goes well, you should get a clear bluish solution.
4. Add 350 µl of COLD Neutralization Buffer (yellow), and immediately cap tube and turn upside down several times to mix. A yellow precipitate should form and there should be no blue color left.
5. Use a disposable pipet to transfer the yellow solution (with precipitate) to a 2 ml filter syringe.
6. Hold the syringe over the miniprep column (in collection tube) and push in plunger. You should get a clear yellow solution, with the precipitate stuck in the filter in the syringe.
7. Place the miniprep column with collection tube in the centrifuge and note the position number of your tube. The TA will operate the centrifuge once all groups are ready.
8. Following centrifugation, recover your miniprep column and collection tube.

9. Transfer your miniprep column to a clean microcentrifuge tube.
10. Carefully pipet 30 µl of Elution Buffer onto the white filter in the miniprep column. Do not poke the filter!
11. Let the column stand for 1 minute. This allows the buffer to thoroughly soak the filter so you elute more DNA.
12. Place the miniprep column and microcentrifuge tube in the centrifuge and note the position number. The TA will operate the centrifuge once all groups are ready.
13. Following centrifugation, recover your microcentrifuge tube with the eluted DNA. Discard the miniprep column.
14. Add 6 µl of DNA Loading Dye to your tube and pipet up and down to thoroughly mix with elutant.
15. Analyze elutant by agarose gel electrophoresis.

Procedure III: Agarose Electrophoresis

You will be analyzing your final products for the PCR and miniprep procedures on agarose gels. The gels contain a dye that will produce a bright green fluorescence under blue light when it associates with DNA. In addition to the PCR and miniprep samples, we will load a DNA standard, or *ladder*, of fragments of known size that you can use to estimate the size of any DNA molecules in your samples.

1. The TA will set up the agarose gel electrophoresis apparatus. This will include an electrode bath (or *gel box*) filled with electrophoresis buffer and a power supply.
2. The TA will place 3-4 agarose gels in the electrode bath in the correct orientation. Note that each gel has eight wells, so it is possible to load one lane with ladder and up to seven lanes of sample on each gel.
3. The TA will load the ladder, demonstrating proper technique for getting samples into the wells.
4. Each group should load three lanes: (i) 15 µl, positive control (pUC19), (ii) 15 µl, PCR product, and (iii) 15 µl, miniprep elutant. Make sure to note which samples are in which lanes.
5. The TA will put the lid on the electrode bath and turn on the power supply. You should see the loading dye moving into the gel within a few minutes.
6. Using the progress of the loading dye as a guide, the TA will turn off the power and take the gels out of the electrode bath when sufficient separation has been achieved.
7. Examine the gel on the transilluminator directly or using a camera. Sketch the results or paste a picture in your notebook.

Notes for the Instructor

These procedures were developed as part of more extensive DNA analysis lab sequence that takes three weeks to complete. The Student Outline contains an abbreviated version of the first exercise in the sequence. The scenario for this lab was originally designed to test for *Salmonella* contamination, but we have modified it so the students are guided to eventually conclude that we have a strain of E. coli harboring a plasmid that at some point acquired a degenerate *Salmonella* sequence and that confers ampicillin resistance. At the end of this exercise as a stand alone unit they would be able to conclude that they do not have *Salmonella* and that the bacteria harbor a plasmid. The expectation would be to get three bands from the PCR if it were *Salmonella*, but we get two bands suggesting it is a "weird *E. coli*" that gives a false positive for one Salmonella marker. Note that as we are not looking to ever get a positive PCR result for *Salmonella* we only include two pairs of primers in our PCR mix. For simulation purposes we use the pUC19 plasmid as "*E. coli* genomic DNA," and pBluescript (pBSII) as our mock *E.coli* with plasmid. Both plasmids are positive for Primers A, our mock enterobacterial marker, but only pBSII is positive for Primers B, our putative *Salmonella* marker. It would be possible include a third plasmid as a mock *Salmonella* sample, as long as it has a sequence not found in the other two (for example, pCRII which has ampR and lacZ, but also kanR).

The goal of this sequence of protocols is to allow novice biology students to perform real molecular biology procedures in an authentic context. A key to the success of this approach has been to streamline the procedures so they can be completed within a three hour lab session. This eliminates the need to run parts of procedures outside of lab or put off completing procedures until the following week, both of which reduce student engagement.

The goal of presenting a realistic approach to a molecular biology problem is accomplished by presenting a realistic scenario involving the identification of bacteria and analysis of antibiotic resistance. We begin by making demonstration plates that test for (1) resistance to ampicillin, and (2) ability to digest lactose. These plates provide an authentic starting point for the colony PCR procedure, and establish the basis for the miniprep procedure.

Our approach to streamlining the following procedures has been to search for the most current protocols and reagents, and then to eliminate steps that are not critical and speed up the remaining steps. While in the research lab we seek to get the best results possible with a minimum of contamination, it the teaching lab we really can use more quick-and-dirty methods that are not perfect but rather good enough. At the same time we continuously strive to reduce student exposure to hazards; here, that has meant finding a safer alternative to ethidium bromide that is robust enough for student use, and the elimination of UV light sources.

Prep Notes I: Bacterial Transformation and Demonstration Plates

In our scenario students are trying to differentiate between *E. coli* and *Salmonella* using different tests. They are also trying to determine if the bacteria are antibiotic resistance, and what the source of that antibiotic resistance may be. The demonstration plates establish that the control bacteria will not grow with ampicillin, but the focal strain will. They also demonstrate that both control and focal strains can digest lactose, in this case using digestion of X-gal which turns colonies blue as a test. The advantage of X-gal plates over MacConkey plates is they can be stored in at 4°C for weeks and the color continues to get darker rather than fading. The ability to digest lactose is a feature used to identify *E. coli* in a clinical setting.

Materials

a. 100 mm bacterial plates, LB/Agarose with Ampicillin (100 µg/ml)
b. Parafilm
c. Sterile spreaders
d. Competent *E. coli* (DH5a)
e. pBluescript plasmid (pBSII)
f. LB, Tryptic Soy or SOC broth

Procedure

1. Dilute pBS II plasmid to 10 µg/ml in sterile water
2. Thaw 100 µl competent *E. coli* on ice
3. Add 10 µl plasmid to cells, and mix by **gently** tapping tube
4. Incubate on ice, 30 min
5. Heat shock cells at 42°C for 60 sec; place **immediately** on ice
6. Add 800 µl broth to cells and mix by inverting tube
7. Incubate at 37°C for 30 min (optional if you have good cells)
8. Plate 100 µl transformed cells on LB/Agarose with ampicillin; incubate at 37°C overnight
9. Seal plates with parafilm; store at 4°C for up to 2-3 weeks; restreak as required

Prep Notes II: Fast Colony PCR

There are three components to this procedure that make it go several times faster than traditional PCR, with total run times < 45 min: (1) Fast polymerase mix, (2) ultra-thin wall PCR tubes, and (3) fast cycling PCR machines. The first two together are sufficient to accelerate PCR even in standard thermocyclers.

Materials

a. Qiagen Fast PCR 2x Master Mix
b. Primer mix (0.5 µM each primer in water)
   - Primers A (*ampR*): tgaggcacctatctcagc-gatct/cgaactggatctcaacagcggtaa
   - Primers B (*lacZ*): atttagagcttgac-ggggaaagcc/accatgattacgccaagctcggaa
c. Sterile toothpicks
d. Ultra-thin wall PCR tubes
e. pUC19 plasmid in water, 10 µg/ml (Mock *E. coli* genomic DNA)
f. Ampicillin resistant bacteria as colonies on plates
g. Tubes of distilled water
h. Microcentrifuge tubes

Procedure

1. Make PCR Master Mix (Complete) by mixing 1 ml Qiagen Fast PCR 2x Master Mix with 200 µl Primer Mix; Students will use 12 µl PCR Master Mix (Complete) plus 8 µl bacterial lysate per group
2. Aliquot 12-15 µl per group, putting total volume for one lab section in each tube
3. Store at -20°C
4. Program PCR machine for a fast PCR run (30-45 minutes.)
   - Step 1 is 95°, 5 min to activate hot-start polymerase
   - Program 20-35 cycles (depending on needed yield)
     - Denature at 95°C, 2-5 seconds
     - Anneal at 55-65°C, 2-5 seconds
     - Extend at 72°C for 10-15 seconds
   - The final step is always a 1 minute extension at 72°C
   - Add 1 minute at 10°C at the end to cool reaction
5. Completed reactions can be stored at -20°C

Prep Notes III: Fast Minipreps

The Zymo Zyppy miniprep procedure eliminates the initial centrifugation to pellet cells, saving a lot of time. The procedure has been further streamlined by using filter syringes to eliminate one centrifugation step, and by completely eliminating the wash steps. This produces a high yield of not-so clean plasmid suitable for electrophoresis.

Materials

a. Zymo Zyppy Plasmid Miniprep Kit (select components can be ordered individually)
   - Spin columns
   - Collection tubes
   - Microcentrifuge tubes
   - 7X Lysis buffer
   - Neutralization buffer
   - Elution buffer
b. 2 ml syringes
c. Kimwipes™
d. Transformed *E. coli* in LB broth (Note: You MUST use LB!)

Procedure

1. Use a colony of transformed cells to inoculate 50 ml of LB + ampicillin (100 µg/ml)
2. Incubate on 200 rpm shaker at 37°C overnight
3. If more cells are needed, use 1 ml culture to inoculate up to 1 liter of broth
4. Aliquot cells in screw cap tubes; 6 ml per lab section
5. Store at 4°C for up to 2 weeks
6. Pack 1/2 Kimwipe into barrel of each syringe with a pipette (or have students do it)

Prep Notes IV: Fast Electrophoresis

The key to fast electrophoresis is using a low-current buffer, such as Sodium Borate or Lithium Borate electrophoresis buffer. This allows gels to run at up to 250V without getting hot, and therefore run twice as fast as traditional TAE or TBE gels which typically run at 70-100V. In addition the use of GRGreen, a room-temperature stable florescent DNA dye, directly in loading buffer allows gels to be immediately examined by students using blue-light transilluminators.

Materials

a. Horizontal electrophoresis chamber
b. 300V Power supply
c. Agarose (Low EEO preferred; NO low melt!)
d. Control plasmid (pUC19 or similar, 10 µg/ml), prestained
e. DNA ladder, wide range, prestained
f. 6X GRGreen loading buffer
g. 1X SBE (Sodium Borate Electrophoresis buffer)
h. Gel casting trays and combs
i. UV or blue-light transilluminator

Procedure

1. Make or purchase 20X SBE stock
   - Available premixed from Lab Supply Mall
   - To make, mix 8 g NaOH and 47 g Boric acid in 1 liter water (final volume)
   - If cloudy, can be vacuum filtered
   - Final pH should be about 8.2
2. Make 4 liters 1X SBE by diluting 20X stock in water
3. Set power supplies to 250V
4. Fill electrophoresis chamber with 1X SBE; change after 3-5 runs

5. Make 0.8% agarose gels with 1X SBE
6. Aliquot 6X GRGreen into microcentrifuge tubes with enough for one lab section
    - Available from Lab Supply Mall
    - Alternatively, us 100X GelGreen or 10X SybrGreen in 6X loading dye
7. Dilute control plasmid to 10 µg/ml; add 6X GRGreen loading dye (to 1x); store at 4°C
8. Add 6X GRGreen (to 1X final) to 6X DNA ladder; store at 4°C

Sources

- Lab Supply Mall, **www.labsupplymall.com** (20X SBE, 6X GRGreen loading dye, blue-light transilluminators)
- Invitrogen, **www.invitrogen.com** (blue LED transilluminators, Sybr Green)
- Phenix Research Products, **www.phenixresearch.com** (GelRed, GelGreen, thin wall PCR tubes (MPC-427Q))

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