PCR on a Roller Coaster

SIGHTINGS

The polymerase chain reaction (PCR) has been a wildy successful and commonly accepted method for "amplifying" sequences of DNA—like a photocopier for molecular biology. It is typically performed in conventional thermal cyclers where, over time, the sample reaction mixture is taken through different temperature states: an annealing stage (~55° to 70°C), an extension stage (~72°C), and a denaturing stage (~94°C). This thermal cycle is repeated about 20 times or more, which takes approximately 1 hour to complete. It turns out that the majority of the time is used for setting the temperature to the correct set point. Manufacturers of thermal cyclers are constantly improving the electronics used for thermal control to try to speed up the process.

A report published last month in this journal describes an apparatus that speeds up the process at least 10-fold (*1*). Instead of using time to cycle the temperature of the reaction mixture, the authors used space by keeping the temperature constant over time at different locations on a glass chip and moving the sample through the individual temperature zones [see figure 1 of Kopp *et al.* (*1*)]. The amount of time required for samples to reach a new temperature depends only on how fast they flow to the next temperature zone and how quickly the heat dissipates. In the system used by Kopp *et al.*, the time needed for heating and cooling the sample is less than 100 ms. As proof of principle, these improvements in time delays allowed the researchers to perform 20 PCR cycles in about 4 min and yield sample quality and quantity similar to conventional thermal cyclers, as determined by slab-gel electrophoresis.

The glass chip is fabricated from Corning 0211 glass and has three well-defined temperature zones kept at 60°, 77°, and 95°C by copper blocks controlled with a thermostat and monitored by digital temperature controllers. The sample is hydrostatically pumped through a single channel (40 μm by 90 μm by 2.2 μm) etched into the glass chip. A glass cover plate is placed on top of the chip, and two precision syringe pumps deliver the sample and the buffer solution through holes drilled in the cover plate, while the product is collected at the outlet capillary. Very small sample volumes, on the order of a few nanoliters, can be run through this system. Because several samples can be run in tandem (provided that some buffer separates the sample plugs), the throughput of a single device can be greatly increased. The authors did not notice any cross contamination between samples, even without major cleaning. They used three different methods to make sure that the sample components (DNA and Taq polymerase) did not stick to the glass capillary walls: the walls of the chip were silanized with dichlorodimethylsilane, a zwitterionic tricine buffer was used, and a nonionic surfactant (Tween20) was added to the PCR mixture.

One drawback of this apparatus is that the thermal cycling is determined by the pattern of the channel passing through the temperature zones, so a new glass chip has to be etched for each cycle pattern. For instance, to perform 40 cycles would require etching a glass chip with 40 revolutions through the temperature zones. The cost for each etched glass chip is $500 and for the entire apparatus is $4,000 (*2*).

Applications of this apparatus may have the greatest impact in the field of medical diagnostics, where quick answers are needed for administration of treatments. For instance, one can envision using this system to determine antibiotic sensitivities in microorganisms instead of starting the patient on a broad-spectrum antibiotic regimen while waiting for bacterial culture and sensitivity results. In the not-so-distant future, individualization of drug therapy based on a patient's genetic make-up and his or her ability to metabolize specific drugs (pharmacogenomics) could also benefit from this speedy apparatus. Finally, this roller coaster–type system can also be applied to other reactions besides PCR, where a significant amount of cycling is required; for instance, this system could be applied to self-activating enzymes or cyclic electrochemical reactions.

–Richard Peters and Robert Sikorski

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Homing Viruses

SIGHTINGS

Everyone who uses mammalian cell cultures must have a silent envy of colleagues who make their living studying processes in simple organisms. Systems like yeast offer robust molecular genetic tools that allow creation of informative mutants at any defined locus. From total gene knockouts to subtle missense alleles to large chromosomal deletions, a genetically manipulatable system can yield a wealth of useful data with a modest amount of effort.

The cellular process that makes targeted genetic manipulation of an organism possible is a high frequency of homologous recombination when exogenous DNA is introduced. In yeast, an experiment can easily be designed in which virtually 100% of all selected events are homologous recombinations. The frequency drops precipitously when one moves up the evolutionary ladder to mammals. In normal human fibroblasts, homologous recombination events can be had at a frequency of only ~1 x 10^{-7} (*1*). In transformed cells (HeLa), the frequency is about the same (*2*). Turning to mice, embryonic stem cells that will target to a frequency of about 1 in 1 million can be isolated. Coupled with a selection, the mouse targeting frequency is high enough to be used for generating novel, genetically manipulated mice.

But many scientific questions would be answered if there was a method for high-frequency introduction of mutations into human cells. Genes in the cell-cycle pathways come to mind as obvious molecules to tamper with. Is there any way to manipulate DNA so that it will home in to its homologous partner after it enters a cell?

The lab of David W. Russell at the University of Washington may now have come to the rescue (*3*). Russell and his colleague Roli Hirata have succeeded in uncovering a peculiar ability of adeno-associated virus (AAV) to increase human gene targeting by orders of magnitude.

AAV is a 4.7-kb single-stranded DNA virus that sometimes integrates into the host genome. Introduction of mutations in the viral Rep protein can cause AAV to integrate randomly at many sites throughout the genome. Given this property, AAV has been used to transduce genes into cells. Curiously, it wasn't until the study by Russell that someone looked carefully with molecular probes to see where these transduced genes were going. Surprisingly, the AAV passenger genes were recombining at a very high frequency with their cellular homologs.

Two genes were studied to measure the rate of homologous recombination with AAV vectors. HeLa cells containing an inactivated [an insertion at base pair (bp) 39] copy of the neomycin-resistance gene were infected with recombinant AAV containing a mutant *neo*^{r} gene that itself contained a different mutation (an insertion at bp 648). Intragenic recombination between these two alleles in vivo would produce an active *neo*^{r} gene, a readout for homologous recombination that confers G418 resistance in mammalian cells. After infection and selection, G418-resistant colonies were screened by Southern (DNA) blot analysis to look for targeted events. Amazingly, simple infection with these high-titer virus constructs yielded as high a rate as one correct targeted event in 500 HeLa cells.

The second locus examined for targeting was the normal, X-linked *HPRT* gene. HPRT-deficient cells are easily selected in medium containing 6-thioguanine, providing a convenient assay for gene targeting at a

single copy locus in male cells. In one series of experiments, an AAV construct containing two *HPRT* exons—one of which contained a 4-bp insertion—was used to transduce three different normal human fibroblast cultures. At high titers, nearly 1% of the total fibroblast population became HPRT-deficient. Molecular analysis confirmed that in the majority of cases, the 4-bp insertion ended up within the endogenous *HPRT* gene, without other mutations or rearrangements.

The use of AAV as a homologous gene delivery vehicle is promising. AAV constructs are easy to manipulate, and the vector is able to transduce large numbers of cells without toxic or cumbersome treatments such as electroporation or microinjection, including in vivo transduction of a wide variety of normal cells in animals. Another advantage is that subtle mutations can be introduced without complicated selection strategies. Obviously, it will be critical to test a wide array of cell lines to see if the effect is widespread and to get a better handle on the reproducibility of the method. However, the demonstration that homologous targeting can now be done rather efficiently in normal human fibroblasts already sets the stage for many experiments. Using PCR, one should be able to efficiently screen a few hundred infected cell culture wells to find desired mutations. Not quite yeast numbers yet, but it's on the right track.

–Robert Sikorski and Richard Peters

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Demystifying Intranets

NET TIPS

The Internet has become a fundamental tool for today's scientist. It is rapidly becoming the preferred method for receiving information from the world at large. Journal articles, meeting schedules, and university documents are the tip of the Internet information iceberg. The Net is great for disseminating information outside of the lab, but can it be harnessed for exchanging information within the lab? Indeed, a private Internet, called an "intranet," can be created within a lab so that lab members can access Web pages and databases that those outside of the lab cannot.

Let's begin with some background. First, remember that the Internet works because each computer has a unique address, called an IP address. To maintain a site on the Internet, you must register your IP address with a central organization, InterNIC. This ensures that all IP numbers will be unique. (To create an intranet, you can modify and use reserved addresses that are not found on the Internet.) Next, note that the language of the Net is called TCP/IP. Third, be aware that most computers built today are easily networked using standard technology known as Ethernet. Fourth, take heart in the fact that most of the software you will need to create an intranet is available for free download now on the Internet (*1*–*3*).

The purpose here is not to give every detail for setting up an intranet, but just to describe the general steps and equipment involved; there are Web sites that provide all of the information you will need (*4*,*5*). To create an intranet, you should have a working knowledge of hypertext markup language (HTML) and familiarity with the basics of your computer environment. If you can install Windows 95 on a computer, you can build an intranet.

As an example, we can create a simple intranet with five PCs running Windows 95. The only hardware requirements for an intranet are that each computer have an Ethernet card installed and that there is a "hub" to connect each computer to each other. Ethernet cards are inexpensive ($50 to $100) and easily installed. (Macintosh users can smile, because Ethernet is a built-in feature.) A simple 8-port hub can cost under $200.

A cable connects each card to the hub (*6*). All computers on the network will then communicate through the hub. Alternatively, you can connect all the cables into built-in Ethernet wall outlets. Most labs will have these outlets, but you should consult with your local systems administrator for guidance in their proper use.

The software requirements for a small intranet are one server and multiple browsers. Select one of the five machines to take on the role of "Web server"; the other four will be "Web clients." On the client machines, simply install the browser of your choice, such as Microsoft Internet Explorer or Netscape Navigator. On the server machine, you will need to install Web server software (*1*–*3*), which actually distributes the pages to the intranet. Although installing a server may sound complicated, setting up a scaled-down Web server, like Microsoft's free Personal Web Server, is very easy. The installation program for this server will be familiar to anyone who has installed typical PC software, and the server configures itself as you install it.

The configuration requirements for an intranet are based on the fact that each computer will need a unique IP address and a common protocol to talk to each other. The language is TCP/IP and it is a built-in feature of Windows 95. The structure of an IP address is such that certain combinations of numbers (for example, 198.168.1.1) are reserved and will not be found on the Internet at large. Choose a reserved number for your base address, then expand on this number to create a unique address for each computer (198.168.1.2, 198.168.1.3, and so forth). Finally, adjust the configurations for the TCP/IP setting in the Windows control panel. We have included instructions for this at www.medsitenavigator.com/tips.

That is it. You now have your own private network for sharing lab data. By creating internal Web pages, you can provide everyone in your group with up-to-date protocols, lab announcements, vendor information, and so forth. It is not that hard to move to the next step and connect your server to a database. The database could be used as a storage area for images, text, or even movie clips. You can even link your intranet to the Internet so that your Web pages can be viewed externally as well. If this is your goal, you will need to learn about things called proxy servers, software that will protect you from security problems that could occur when your computer is exposed on the Internet. If you choose this route, there is a simple (although not free) server called Rideway (www.itserv.com) that can be set up in a few minutes.

–Robert Sikorski and Richard Peters

Notes

1. Windows 95 personal Web server software is available from www.microsoft.com/ie/pws/default.htm or www.omnicron.ab.ca/httpd/index.html, along with instructions for installation.
2. Macintosh server software is available from www.microsoft.com/ie/mac/pws/default.htm, along with instructions for installation.
3. Server software for UNIX, Windows-NT, and several other operating systems is available from www.apache.org
4. Information on setting up an intranet is available at http://207.68.156.54/office/intranet/intranetwp/default.htm and http://207.68.156.54/intranet/
5. Information on intranets for office computing is available at http://www1.zdnet.com/pccomp/oc/projects/0198proj.html
6. Information on building an intranet at home is available from http://cnet.com/Content/Features/Howto/HomeLAN/index.html

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