Mass Spectroscopic Genomics

SIGHTINGS

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry is a technique that detects molecules on the basis of an intrinsic physical property of a molecule—its mass-to-charge ratio. Because there is no need for gel electrophoresis and radioactive or fluorescent labeling, the method provides an alternative approach for the analysis of nucleic acids. The method has been coupled with solid-phase affinity methods to increase the speed at which the technique can be used as a large-scale detection tool. The method has been previously used, with DNA duplex probes, to analyze genetic variations in DNA (*1*).

A recent report by Griffin *et al.* extends this approach by means of peptide nucleic acids (PNAs) rather than DNA duplexes as the DNA probe (*2*). The reasons for switching to the use of PNAs are that they offer increased stability and specificity. In addition, they also can hybridize at low salt concentrations and give a better signal in MALDI-TOF mass spectrometry.

The authors first had to design a set of test PNA probes. They chose to study the natural genetic polymorphism of the human tyrosinase gene where there are four common polymorphic sites. Two allele-specific PNA probes were synthesized: one complementary to the wild-type allele and one complementary to the single base substitution variant. In order to give each of these probes a unique mass that could be detected by the mass spectrometer, each was attached to different numbers of mass labels. The mass label they used was 8-amino-3,6-dioxaoctanoic acid molecules, which can be covalently attached to the NH_2-terminal end of the PNA. Once the probes have been synthesized, they are hybridized with polymerase chain reaction products that have been biotinylated and immobilized on streptavidin-coated magnetic beads. After washing the samples, the beads are applied to the MALDI probe tip. A chemical matrix is added and is allowed to crystallize, leading to the dissociation of the hybridized PNA probe while the bead with its DNA target remains on the probe tip. MALDI of the dissociated PNA probe is then performed, and the signal is detected by TOF mass spectrometry.

The approach was able to unambiguously analyze multiple polymorphic sites for tyrosinase on human DNA samples and was able to identify heterozygotes. The PNA probes provided a high degree of specificity. In addition, they could hybridize in the absence of salt, which decreases the likelihood of formation of secondary structure in the DNA strand immobilized on the magnetic bead. In addition, this lack of salt avoids the need for additional washing steps that are often required to remove unwanted salts in DNA-based analyses.

There are, however, two major drawbacks that will need to be resolved before this method can be applied to the large-scale detection of genomic variations. First, the PNA probes had different hybridization stabilities, so they could not be combined in the same reaction mixture. Each set of probes needs different wash conditions in order to get good discrimination between the wild-type and variant probe. Thus, in the tyrosinase example, four separate reaction mixtures were required. The second drawback is that various PNA probes have different mass spectral intensities. The authors were not able to find conditions that would allow satisfactory signals from all the probes when mixed together. Thus, each spectrum had to be acquired separately, and a final composite spectrum had to be created.

So, before the above approach can reliably be applied to high-throughput screening of genetic polymorphism, more needs to be understood about PNA probes: what predicts their stability with DNA duplexes, and what determines their signal intensity in MALDI-TOF mass spectrometry. However, use of the digital readout produced by mass spectroscopic methodology is still very promising for future genomic applications.

–Richard Peters and Robert Sikorski

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Molecular Plumbing

SIGHTINGS

The goal of tissue engineering is essentially to create "replacement parts" that can be grafted into humans. The utility of universal grafts—namely, tissue that is accepted by any individual in any part of the body—would be enormous. In the case of vascular surgery, for instance, tailor-made vascular grafts would provide a critical supply of vessels that could be used in surgery. To date, the strategies for tissue engineering have usually relied on the availability of various synthetic materials to provide mechanical strength and scaffolding to cultured cells.

The most significant challenge facing vascular transplantation is that small-diameter biosynthetic grafts invariably show a poor patency rate (that is, a high rate of vessel collapse). Chronic inflammation from low-level graft rejection of the inert matrix is a major contributing factor to the closing up, or stenosis, of the grafts. In addition, the presence of the graft as a foreign body increases the likelihood of infection. Attempts at creating a completely biologic blood vessel in vitro without any synthetic scaffold have been successful, but the resultant vessels lacked the necessary strength required to function in vivo (*1*). More recently, a completely biologic, autologous (that is, taken from the same individual) vascular graft was generated using the small intestine submucosa in a dog model (*2*). Unfortunately, experiments with human intestinal tissue failed to show the same effect.

Now comes some good news for all those would-be molecular surgeons. A team of scientists has just reported a method to generate a completely biological, tissue-engineered human blood vessel (*3*). The blood vessels, generated in vitro, are devoid of any synthetic material. Moreover, these tissue-engineered vessels were organized into a well-defined, three-layered histological architecture, complete with an intima, a media, and an adventitia (inner, middle, and outer layer). Like natural blood vessels, the man-made endothelium was confluent, expressed von Willebrand factor, incorporated acetylated low-density lipoprotein, produced prostacyclin (PGI2), and inhibited platelet adhesion. Amazingly, smooth-muscle cells reexpressed desmin, a marker normally lost under standard culture conditions. Collagenase activity was also kept to a minimum in this engineered vessel. Impressively, the burst strength (the hydrostatic pressure required to burst the vessel) was actually higher than the burst strength of the large saphenous veins of the leg, harvested from patients undergoing vascular reconstruction. How did they do it?

Their secret was to capitalize on the increased synthesis of extracellular matrix by mesenchymal cells when they are grown in the presence of ascorbic acid. It is this increase of the density of extracellular matrix that provides increased burst strength to this vessel. Here is a step-by-step description of the approach used by the scientists.

Endothelial cells were plated in gelatin-coated culture flasks; smooth-muscle cells and fibroblasts were cultured in separate flasks, each supplemented with ascorbic acid (50 μg/ml). After 1 month, these cells formed sheets (cells plus extracellular matrix) that could be peeled off the flask.

Adventitias from fibroblast cultures were dehydrated and used to provide an acellular inner membrane. This inner membrane was slipped around a perforated polytetrafluoroethylene tube, and a sheet of smooth-muscle cells was rolled around it to produce the vascular media. This construct was placed for 1 week in a bioreactor apparatus that provided mechanical support and luminal flow of cul-

ture medium. Next, a sheet of fibroblasts was rolled onto the outside to provide the adventitia. After a maturation period of 2 months, the inner tubular mandrel was removed, and endothelial cells were seeded in the lumen. All culture media contained ascorbic acid until seeding of the endothelial cells. In total, the production of the graft takes 3 months of culture.

The authors tested the in vivo graftability of this construct by implanting it in dogs. To avoid acute rejection of the xenograft, the implant was not endothelialized. The grafts withstood surgical handling and in vivo hydrostatic pressures. However, there was a high rate of graft failure due to thrombosis, as would be expected of a vessel lacking endothelial lining. The construction of a similar dog-engineered blood vessel is needed to test the long-term value of this synthetic graft in an animal model.

Clearly, this method opens up the door for interesting therapeutic opportunities such as the generation of custom-made vascular grafts or the use of these grafts as gene therapy "sanctuaries." In the future, one can imagine assembling these vessels with other custom-made tissues to make de novo organs. Before this happens, researchers will first need to reduce the time needed to generate the vessels in order to apply this approach to elective vascular reconstruction. Also, the model will have to be adapted to generate vessels with a length of 15 cm or longer so they can be used in vascular reconstruction.

Although physicians will have to wait for clinical applications, scientists interested in studying the vascular cells or in experimenting with the effect of physical forces on them could begin working at once with this very realistic human vessel model.

–Richard Peters and Robert Sikorski

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On the PROW

SITE FINDER

Protein Reviews on the Web (PROW; www.ncbi.nlm.nih.gov/prow/) is an online knowledge environment that organizes human protein and gene information. As a proof of principle, the editors of PROW have first tackled human cell-surface molecules (CD). There are approximately 200 human CD cell-surface molecules, and information about each of them is available on this Web site.

The content on PROW is a combination of "PROW Guides," peer reviewed contributions from selected experts in the field and "Forum entries," which are open World Wide Web submissions. The latter are reviewed by editors before appearing on the site. PROW can be searched by entering query terms or viewing an index. For each molecule, short reviews are available in bullet format.

Hypertext links to other Web resources such as SwissProt, Mendelian Inheritance in Man, and Medline/PubMed are available to add more depth to the content. Viewers are encouraged to provide feedback by adding comments that can be linked to the review. In principle, the PROW approach can be applied to other families of molecules.

The open, global approach of PROW is a highly laudable one, but for it and other similar Web-based projects to be sustainable, contributions from many authors will be essential. Over time, it may be hard to solicit submissions on goodwill alone. Perhaps academic tenure boards will start taking into consideration an author's track record for publishing in sites such as PROW.

–Richard Peters and Robert Sikorski

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The X Files

NET TIPS

How often do you receive a file attached to an e-mail message only to find out you can't open it, or, if you open it, you can't read it? The problem arises usually for one of two reasons. The first case is that the file was compressed during the sending process, and your system lacks the necessary utility to uncompress the file. The other case is that you do not have the necessary application installed on your computer to read the file, even if it is not compressed. Indeed, with all the software applications floating around, it is often difficult to know what to do with the file you have received if you don't have the particular application used for creating the file. Here is a step-by-step approach to solve file attachment problems: First, if you do not have a compression/decompression utility installed on your system, get one. In today's e-mail/Internet world, this is an absolute must. Compressed files come in various "flavors," characterized by an extension, as in FILENAME.EXT, where EXT is the extension. The following is a list of the most common compressed file formats with their extension code in parentheses: StuffIt (.sit), Compact Pro (.cpt), AppleLink (.pkg), ZIP (.zip), ARC (.arc), gzip (.gz), tar (.tar), UNIX Compress (.Z), LHA (.lha), uuencode (.uu), BinHex (.hqx), MacBinary (.bin), and Base64/MIME (which is internal to e-mail systems). To uncompress these types of files on the PC, we suggest using WinZIP. It is an acclaimed compression/decompression utility for the PC which can handle popular file formats. Version 6.3 has recently been released, and a free evaluation copy can be downloaded from the Web at www.winzip.com/.

For the Mac, we suggest StuffIt Deluxe. This commercial product is the most popular compression/decompression and file decoding utility for the Mac, and it too can handle almost any file format. A free "Lite" version is available, but it does not possess all the muscle power of the commercial version (www.aladdinsys.com/deluxe/index.html).

Once you have been able to decompress the file, check to see if you have the application that created it. For instance, use Microsoft Word to open a Word file. If you do not have the application, try using a similar application: They often have conversion scripts built into them. For instance, you can usually read a Word file by using WordPerfect. If that does not work, you may need a file conversion program. This utility is software that lets you view files and convert them to a different format that you can edit on your system. PC users would be well served by using Conversions Plus. You can view almost any file by using this utility on a PC (www.dataviz.com/Products/CPW/CPW_Home.html). For Mac users, try using StuffIt Deluxe, because it has a file conversion utility bundled with its decompression utility. Finally, if everything else fails, go on the Internet to find a file conversion specific to that file format. There are a countless number of them for various applications and data formats, such as the ones for the various versions of Word (www.microsoft.com). We have listed a few key places to look at www.medsitenavigator.com/tips.

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