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Prime time for real-time PCR

Real-time PCR is the favored method for measuring gene expression. Researchers benefit from a vast and growing choice of reagents and instruments for their experiments. Laura Bonetta reports.

Real-time PCR combines the amplification of a DNA sequence with the detection of the amplified products during each reaction cycle—in other words, in real time. In comparison to conventional PCR, it can be used to detect a much wider range, over 10^7 fold, of starting template concentration. It is also less time-consuming, as it does not require analysis of the end products by gel electrophoresis, and can provide a quantitative result. It is no surprise that the technique, first described in the mid-1990s, has quickly grown in popularity. Researchers use it to measure gene expression and copy number, calculate viral titers, and carry out single-nucleotide polymorphism analysis, to mention just a few applications.

In particular, real-time reverse transcription (RT)-PCR has become the method of choice to rapidly and quantitatively examine the expression of specific genes. Scientists can readily detect as little as a twofold change in the expression of a target gene in response to different treatments in hundreds of samples per day.

Because of the prominence of real-time PCR, companies are flooding the market with newer, improved reagents for every step of the process, from sample preparation to reverse transcription to amplification—all of which promise to make the process even faster, more efficient and more reliable (see **Box 1**). In addition, the methods and instruments used to measure the amplification products continue to become more sophisticated. As a result, researchers wanting to use real-time PCR are faced with a staggering choice of options.

PCR gets real

PCR was developed in 1983, a discovery that earned Kary Mullis the Nobel Prize in Chemistry ten years later. The technique, now a staple of every molecular biology



Real-time PCR reagents. (Courtesy of Applied Biosystems Group.)

lab, uses the thermostable *Taq* DNA polymerase to extend short single-stranded synthetic primers using the target DNA or cDNA as a template during repeated cycles of heat denaturation, primer annealing and primer extension. With each cycle the amount of template DNA is doubled until one of the reagents becomes limiting, and the reaction reaches a plateau. At the completion of the reaction, amplification products are analyzed by size fractionation using gel electrophoresis.

Real-time PCR follows the same course, except that the products are detected as they are made, during the exponential phase of the reaction rather than at the end. Detection reagents now on the market are based on probes and dyes that produce a fluorescent signal each time a double-stranded product is made.

The more copies of nucleic acid present at the start of the reaction, the fewer amplification cycles are required to make

sufficient product to detect by fluorescence imaging. The cycle in which a significant increase in fluorescence above the threshold is measured—referred to as the C_T value—can therefore be used to calculate the quantity of DNA in the sample.

Detecting amplicons

To carry out real-time PCR, researchers have to choose not only what primers to design (see **Box 2**) but also what detection chemistry to use. In many cases these decisions will be influenced, if not determined, by the thermocycler (see **Box 3**) that they have access to and the instrument's chemistry and dye compatibilities.

There are many popular chemistries for real-time PCR. One class uses different fluorescent dyes incorporated in short oligonucleotide probes specific for the amplified target. The second class consists of dyes that bind double-stranded DNA and become fluorescent; the most

commonly used of these is SYBR Green I, sold by many companies that provide PCR reagents. As the amount of PCR product increases, more SYBR Green I dye binds to DNA, resulting in a steady increase in fluorescence. The technique is inexpensive and generic, as it requires the same detection reagent for each template to be tested. But detection with dyes like SYBR Green I is less specific than probe-based detection methods. For example, if primers bind to each other, the dye will bind to these so-called primer dimers and generate a signal. In addition, SYBR Green I cannot be used in multiplexed assays—in which several distinct targets are included in a single

tube or well—because it will not distinguish among different sequences.

Despite these drawbacks, SYBR Green I can be used to quantify the amount of template in a sample if the PCR is fully optimized. But many researchers prefer to use this dye to optimize PCR and check that the primers are working well, before ordering a probe-based assay.

TaqMan rules

A variety of probes specific for the amplified target (or amplicon) can be used in real-time PCR. By far the favorite, especially among scientists who have just started using the technique, are TaqMan probes.

BOX 1 SOME LIKE IT HOT, SOME FAST

In the beginning, for those wanting to do PCR, there was *Taq*. Nowadays thermostable DNA polymerases come in different flavors, each with its own unique capabilities. Researchers also benefit from a variety of kits for amplification, reverse transcription and sample preparation. Some master mixes are optimized for fast reactions, whereas others contain proprietary reagents that prevent mispriming.

Many of the commercially available *Taq* polymerases are special blends, such as Fermentas' high-fidelity PCR enzyme mix. Stratagene markets the *Pfu* polymerase, which has a lower error rate than *Taq* thanks to its proofreading capacity. Takara Mirus Bio, on the other hand, sells *Bca* BEST, a DNA polymerase with strand-displacing and template-switching activities that can be used to perform reverse transcription and DNA amplification in a single tube.

So-called hot-start polymerases are variations of the naturally occurring enzyme that become active only at high temperatures (typically 95 °C). This property reduces the chances of mispriming—that is, the polymerase extending primers bound to complementary or partially complementary sequences on nontarget DNAs while the reaction is being set up. “Products created during setup decrease the efficiency of PCR,” says Joseph Donnenhoffer of Roche. The company's FastStart *Taq* polymerase has a one-base deletion that renders the enzyme inactive at room temperature. The polymerase is sold as part of the LightCycler real-time PCR kits. Sigma Aldrich sells a *Taq* polymerase that is supplied inactivated by an antibody, which is then released by heat.

In addition to making the amplification step more effective, companies are also developing methods to make sample isolation more straightforward and less error-prone. Cepheid's GeneXpert kit integrates sample preparation with real-time PCR. “You can load the sample directly into a cartridge, which is then inserted into a GeneXpert module. It is the technology currently used by the US post office for monitoring the presence of biothreat agents in air samples,” says Bill McMillan.

Ambion sells a Cells to Signal kit that produces a sample ready for PCR in 5 minutes. “It is the first of its kind,” says Christy O'grea, associate product manager. You can skip the RNA isolation and do the reverse transcription in a crude cell lysate so that there is no loss of template. It can be used for cultured cells and primary cells alike.”



Each TaqMan probe consists of a single-stranded oligonucleotide that is complementary to a sequence within the target template. It has a fluorescent dye at its 5' end, whose signal is stifled by a quencher moiety at the 3' end. Soon after the TaqMan probe hybridizes to one of the strands on the template, it is digested by the exonuclease activity of the *Taq* DNA polymerase as it extends the amplification primers. Chopping up the probe releases the fluorescent dye from the quencher, resulting in an irreversible increase in the fluorescence signal.

Applied Biosystems Group (ABI) currently offers “over 200,000 TaqMan Gene Expression Assays for human genes and their variants,” says Kelly McDonald, senior scientist at ABI. An advance in the technology has been the addition of a DNA minor groove-binding (MGB) moiety at the 3' end of the TaqMan probes, increasing their stability and specificity. This modification allows the probes to be shorter—about 13–20 base pairs (bp) long—and thus “cheaper to make and

more flexible to move where you want within the gene,” says McDonald. Another company, Epoch, produces its own brand of MGB probes called MGB Eclipse.

ABI has exploited the high specificity and sensitivity of the TaqMan assays to develop a method to specifically amplify and quantify microRNAs (miRNAs), which are short endogenous RNAs believed to play primary roles in gene regulation¹. ABI's new line of assays uses special looped primers for reverse transcription of the miRNAs; the resulting cDNAs are then amplified by real-time PCR. These assays can be used to quantify miRNA levels and discriminate between mature miRNAs and their precursors.

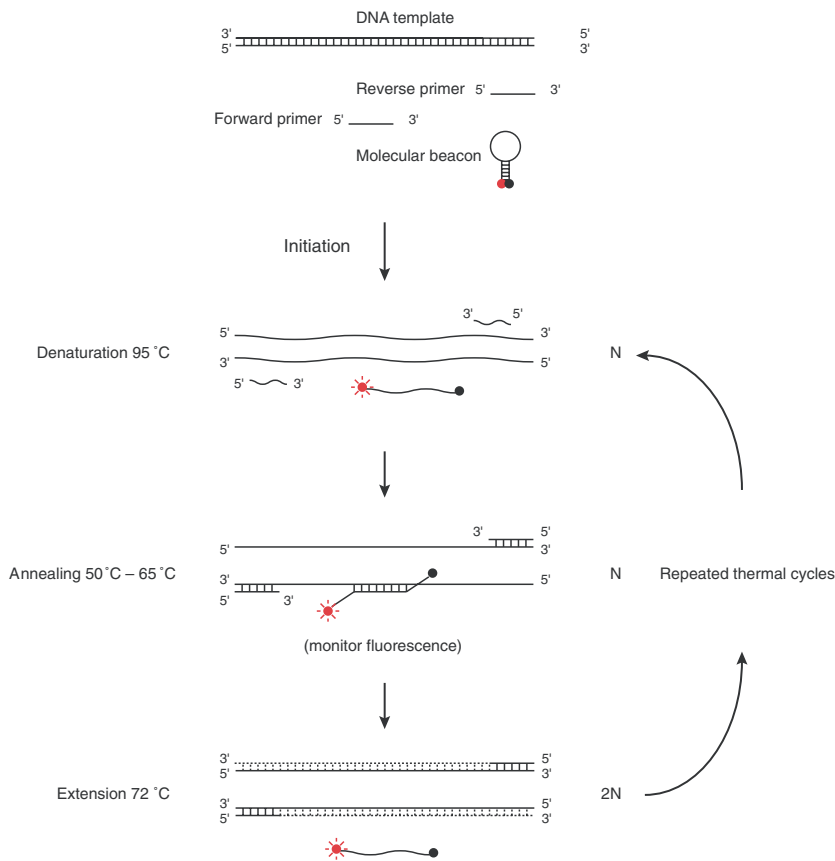
“We currently have close to 200 validated assays for human miRNAs. They can quantitate miRNAs with a broad range of expression levels, starting from as little as 1 ng of total RNA,” says McDonald. “The assay can be done directly on whole cells or cell lysates to save time and eliminate the risk of losing the miRNAs during the isolation process.” ABI plans to expand

its product portfolio to include similar assays for the detection of short interfering RNAs (siRNAs)—RNA molecules used to knock down the expression of specific genes¹. “These assays can be used to validate whether a siRNA is efficiently transfected into cells, as well as monitor the molecule's lifespan,” says McDonald. ABI plans to introduce its preliminary miRNA assays through an early access program this spring.

Tighter binding

Exiqon developed a line of probes incorporating locked nucleic acids (LNAs) that can be integrated into different types of probes, including TaqMan and molecular beacons, to increase the stability of the probe-target duplex. LNA-containing probes can be even shorter than MGB probes and offer greater flexibility in design.

Exiqon has developed a ProbeLibrary of 90 prevalidated real-time detection probes substituted with LNAs that can recognize 98% of transcripts cataloged in the RefSeq database at the National



Real-time PCR detection using molecular beacons. (Courtesy of Salvatore Marras, Public Health Research Institute.)

Center for Biotechnology Information. The reason these probes can recognize such a high number of transcripts is that each probe is only 8–9 nucleotides long and can therefore detect more than one transcript. Assays specific for a particular target can be designed by using the right primer-probe combination.

To help with this task, Exiqon has launched a free online software product to design primers for a particular transcript, usually spanning the exon-exon splice junctions, and then identify one or more LNA probes that will detect the amplification product. “A big issue in real-time PCR is the time spent on assay design,” says Peter Roberts, sales manager for Exiqon. “A typical design will use up most of the morning for a researcher. Our software does it in seconds.” By combining individual Probelibrary probes and target-specific PCR primers selected using the software, Exiqon’s Assay Design Center (<http://www.probelibrary.com/>) can be used to create more than 644,000 distinct assays for the human transcriptome. “It is the

only commercially available system that provides specific kits for real-time PCR for model organisms such as *Arabidopsis*, *Drosophila* and *C. elegans*, in addition to the usual human, mouse and rat,” says Roberts.

Hairpins and more hairpins

Several chemistries developed after the TaqMan probes flaunt a hairpin loop structure. Molecular beacons², first developed at the Public Health Research Institute (PHRI) in Newark, New Jersey, consist of a single-stranded loop complementary to the target template and a double-stranded stem, about six bases in length, with a fluorophore at one end and a quencher at the other end. When the probe is in a hairpin configuration, the fluorophore and quencher are in close proximity. But the probes are designed in such a way that they will bind to the amplicon at a specified temperature—because the probe-target duplex is thermodynamically more stable than the hairpin structure. Upon binding, the stem comes apart and the fluorophore

and quencher are separated, giving off fluorescence. “These probes are very flexible and can be used for any existing PCR application and machine,” says Salvatore Marras, one of the developers of this technology.

Because the stem structure does not participate in the hybridization reaction itself, its length can be adjusted to add specificity to the probe. “Once you have the desired target you can optimize the primers and hybridization temperature to create the most optimal thermal cycle conditions for the PCR, and then adjust the structure of the molecular beacons so that they will only hybridize to the amplicon at that temperature,” says Marras.

Unlike with TaqMan probes, the fluorescence produced at each amplification cycle is reversible, as the probe is not destroyed, resulting in a lower overall background.

“You can use six or seven different molecular beacons in the same tube because of the low background,” says Marras, whose group has also designed assays that can identify 15 different targets in one tube. “In one assay a particular target will be detected as a combination of two fluorophores. If you use a machine that can detect six colors, you could use 15 pairs of molecular beacons—each pair specific for one target but labeled with a combination of two different fluorophores—in the same tube and look for different signatures,” says Marras.

Marras has also used molecular beacons synthesized using 2'-O-methyl RNA, which are nuclease resistant and have higher affinity for RNA, to measure the formation of RNA transcripts in a test tube³. “We wanted to study inhibitors of RNA polymerase, many of which are antibacterial drugs currently on the market,” says Marras. “We designed molecular beacons to bind to transcripts that are formed when an RNA polymerase transcribes from an artificial DNA template. The system allows us to follow transcription in real time. We could potentially screen through thousands of inhibitors and look not only at the level of inhibition but also at the kinetics.”

Another chemistry that uses hairpin structure is that of Scorpions, first developed by David Whitcombe at DxS⁴. Unlike beacons, Scorpions are PCR primer and probe in one. They consist of a primer that has a 5' stem-loop tail with a fluorophore and a quencher in close proximity. This ‘tail’ will hybridize to the extension product of

the primer, thereby releasing a fluorescent signal. "For TaqMan and molecular beacons, the target and probe have to collide to have a signal, but with Scorpions you have everything on the same molecule," says Stephen Little, DxS CEO.

As a result, Scorpions have a fast reaction mechanism. "A probe cannot bind to its target if the target amplicon hybridizes to itself, for example if it contains repetitive sequences. But this is not a problem with Scorpions because they hybridize so quickly after the amplicon is produced," says Little. "They are well suited for problem sequences."

New kid on the block

Promega has developed a new chemistry that lies between the nonspecific detection using SYBR Green I and the probe-based assay. The soon-to-be-released Plexor system uses certain modified nucleotides that are recognized by DNA polymerase but do not form duplexes with normal, unmodified nucleotides. In a Plexor reaction, one

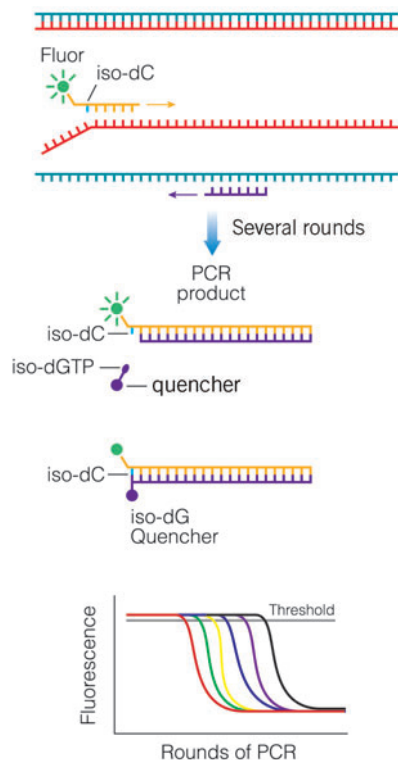
BOX 2 GETTING PRIMED AND PROBED

Many companies provide internet-based tools to help researchers design PCR primers and probes. But a new service provided by the National Cancer Institute will do the work for you. The Quantitative PCR Primer Database (<http://web.ncifcrf.gov/rtp/gel/primerdb>) profiles more than 3,000 published primers and probes that can be used to quantify human and mouse mRNA by RT-PCR. Users can search the database by gene, species or type of assay (TaqMan, SYBR Green I or RT-PCR) to find the primer's location, sequence and original reference. Similarly, the RTPrimerDB database (<http://medgen.ugent.be/rtpprimerdb>) provided by Ghent University in Belgium contains lists of real-time PCR primers and probes for SYBR Green I, TaqMan, hybridization and molecular beacon assays.

oligoprimers for the PCR are tagged at the 5' end with the modified nucleotide and a fluorophore. The reaction is then carried out in the presence of the four natural nucleotides and a modified nucleotide (complementary to the one at the 5' end of the primer) that has a quencher moiety attached. As the PCR proceeds and the target is amplified, the quencher winds up base-paired to the fluorophore. Thus,

the Plexor system measures a decrease in fluorescence as the reaction proceeds. "But the quantification is calculated exactly the same as with TaqMan," says Gary Madsen of Promega.

The technology is similar to detection with SYBR Green I in its simplicity, but allows multiplexing. To help researchers configure single- or multiplex assays, the Promega website will have a Plexor



Overview of the core technology in Plexor Systems for qPCR and qRT-PCR. (Courtesy of Promega.)

the target and one for the control,” says Madsen. “Plexor allows you to do things faster and more accurately.” The company plans to launch the product in May 2005.

Standards, standards

Regardless of the detection method used, researchers calculate the quantity of a target gene or transcript in a sample from the C_T value obtained during the PCR. The calculation can be done in several ways, either by using a standard curve of C_T values obtained from a serially diluted standard solution (absolute quantification) or by comparing the difference in the C_T values of two samples (relative quantification).

As the name implies, absolute quantification results in an actual number of DNA molecules in a sample, but many experts caution that such claims should be treated with a dose of skepticism. “I always tell customers I quantify relative to the standard I am using. There is really no absolute quantification,” says Gregory L. Shipley, director of the Quantitative Genomics Core Laboratory at the University of Texas Health Science Center in Houston, who provides real-time PCR as a service to academic labs. But for most basic research applications, absolute quantification may

not be a necessity. “Usually you only want to have a relative measure. For example, you want to see a gene go up relative to another,” says Shipley.

Real-time PCR is a relatively easy technique to learn, and as new, cheaper instruments and off-the-shelf kits come on the market, many labs are embracing it as a valuable tool in their research. But the apparent accessibility of the technique may be somewhat misleading. “The trick is not the PCR or detection. With most probes you can detect as few as 10 to 1 copies of target DNA and about 100–200 copies for RT-PCR,” says Shipley. “But the problem is sample purification, the method used for the reverse transcription, the choice of internal controls and standards.” The deluge of products and kits that researchers have at their hands can make it difficult to compare the results of one experiment to another. As a result, discussions among experts in the field often center on the issue of standardized protocols and controls to use in real-time PCR experiments.

Better LATE?

Another property of PCR that can complicate quantification is that it is an exponential rather than a linear reaction. This means that a change in amplification efficiency between samples translates into a huge difference in results when the samples are amplified through dozens of doublings. The problem is particularly

Primer Design tool that will recommend the primer sets, including fluorophores, to be ordered for one or several target genes. One advantage of multiplexing is that it allows researchers to run the target and internal control in the same well. “Right now most researchers will do one assay for

BOX 3 THERMOCYCLERS GO REAL-TIME

Many thermocyclers currently on the market are real-time instruments—in other words, they monitor the accumulation of PCR product in real time and automatically analyze the data. They vary in sample capacity—from high-throughput instruments to handhelds—fluorescent dye flexibility, speed and of course price.

Roche’s LightCycler—a popular machine among scientists—can run a PCR in 16–30 minutes. Instead of using plastic tubes and plates, it uses glass capillaries. The machine was originally set up for use with Roche’s own brand of hybridization probes, but it will accommodate other chemistries, such as molecular beacons and Scorpions.

Cepheid’s Smartcyler is also a very fast machine. One of its unique features is that it contains up to six processing blocks, each with 16 independently programmable modules that perform four-color real-time PCR. The blocks can be linked together for high-throughput applications or kept separate

so that multiple users can simultaneously carry out individual experiments, each with a distinct protocol. “It is not a batch machine. A user can run six or seven samples, and while those are running start another independent reaction,” says Bill McMillan.

ABI recently released its 9800 Fast Thermal cycle, a high-speed version of its 7900 thermocycler for high-throughput applications. “It has the most features and the highest capability,” says Andi Felton. “It can use the fast block, regular 96- and 384-well plates, and the low-density array block.” In January of this year, ABI also released a high-speed version of its 7500 platform for medium- to low-throughput applications. The two instruments differ in the types of fluorophores they can support.

Other popular machines for real-time PCR include Bio-Rad’s iCycler IQ, a 96-well machine that can detect four dyes, and Stratagene’s Mx3000P, a lower-cost personal thermocycler.

important when the reaction uses very little template DNA.

“When you use small starting samples you can run up against real problems,” says Lawrence Wangh of Brandeis University, who developed a new method for real-time quantitative analysis of RNA called linear-after-the-exponential (LATE)-PCR⁵. “When using real-time PCR to quantitate low levels of starting materials, one of the problems researchers can encounter is more scatter among replicates. This is because the more cycles you need to get a fluorescent signal, the more opportunity to get an error,” says Wangh. “Another problem is that you can get mispriming, leading to changes in reaction efficiency. If the

amplification reaction does not start in the first cycle, it can result in one C_T shift.”

Wangh’s method uses primers at two different concentrations—a limiting concentration and an excess one—so that when the limiting primer is used up, the reaction proceeds in a linear fashion. A key feature of the technique is that the melting temperature of the limiting primer is equal to or greater than that of the excess primer. “This means that we can shift the annealing temperature up. This makes the reaction more stringent and cleaner, without affecting efficiency. This leads to less scatter among replicates and a stronger signal,” says Wangh.

LATE-PCR is just one example of the developments in the PCR field. As methods

to quantify gene expression by real-time PCR become more sophisticated and more products and reagents become available to researchers, real-time PCR is poised to become a prominent tool for scientific research in the genomics information era.

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SUPPLIERS GUIDE: COMPANIES OFFERING PCR REAGENTS AND INSTRUMENTATION

Company	Web address	Company	Web address
Abgene	http://www.abgene.com/	GenoMechanix	http://www.geno-mechanix.com/
Active Motif	http://www.activemotif.com/	GeneWorks	http://www.geneworks.com.au/
Agilent Technologies	http://www.home.agilent.com/	Hy-labs	http://www.hylabs.co.il/
AlleLogic Biosciences Corp.	http://www.allelogic.com/	Idaho Technology Inc.	http://www.idahotech.com/
Ambion	http://www.ambion.com/	Integrated DNA Technologies	http://www.idtdna.com/
Apogent	http://www.apogent.com	Invitrogen	http://www.invitrogen.com/
Applied Biosystems	http://www.appliedbiosystems.com/	Lark Technologies	http://www.lark.com/
Artus Biotech	http://www.artus-biotech2.de/	LGC	http://www.lgc.co.uk/
Biochain	http://www.biochain.com/biochain/	Lucigen	http://www.lucigen.com/
Biogene	http://www.biogene.com/welcome.cfm/	Maxim Biotech Inc.	http://www.maximbio.com/
BioHelix Corp. (NEB)	http://www.biohelix.com	MJ Research (Bio-Rad)	http://www.mjr.com/
Bioneer	http://www.bioneer.com/	Midwest Scientific	http://www.midsci.com/
Bio-Rad Laboratories	http://www.bio-rad.com/	Molecular Probes (Invitrogen)	http://www.probes.com/
Biosearch Technologies	http://www.biosearchtech.com	MWG Biotech	http://www.mwg-biotech.com/
Biosource	http://www.biosource.com/	New England BioLabs	http://www.neb.com/
Biotrove	http://www.biotrove.com/	Novagen (EMD Biosciences)	http://www.emdbiosciences.com/
Bio S&T	http://www.biost.com/	PGC Scientifics	http://www.pgcscientifics.com/
Brinkmann Instruments	http://www.brinkmann.com/	PerkinElmer	http://las.perkinelmer.com/
Chemicon International	http://www.chemicon.com/	Premier Biosoft International	http://www.premierbiosoft.com
Cepheid	http://www.cepheid.com/	Promega	http://www.promega.com/
Corbett Research	http://www.corbettresearch.com/index2.html	Qiagen	http://www1.qiagen.com/
DxS Limited	http://www.dxsgenotyping.com/	Roche Applied Science	http://www.roche-applied-science.com/
Epicentre	http://www.epicentre.com/	Seegene	http://www.seegene.com/
Epoch Biosciences	http://www.epochbio.com/	Serologicals Corporation	http://www.intergenico.com/
Eurogentec	http://www.eurogentec.be/	Sigma-Aldrich	http://www.sigmaaldrich.com/
Exiqon	http://www.probelibrary.com/	Sigma-Genosys	http://www.sigma-genosys.com/
Fermentas Inc.	http://www.fermentas.com/	Stratagene	http://www.stratagene.com/
Finnzymes	http://www.finnzymes.com/	SuperArray Bioscience Corp.	http://www.superarray.com/
Fluorescentric	http://www.fluorescentric.com/	Synthegen	http://synthegen.com
Genetic Research Instruments	http://www.gri.co.uk/	Takara Mirus Bio	http://www.takaramirusbio.com/
Genetix	http://www.genetix.com/	Thermo Electron Corporation	http://www.thermo.com/
Gene Therapy Systems, Inc.	http://www.genetherapysystems.com/	Techne	http://www.techne.com/
GenScript	http://www.genscript.com/	USB Corp.	http://www.usbweb.com/