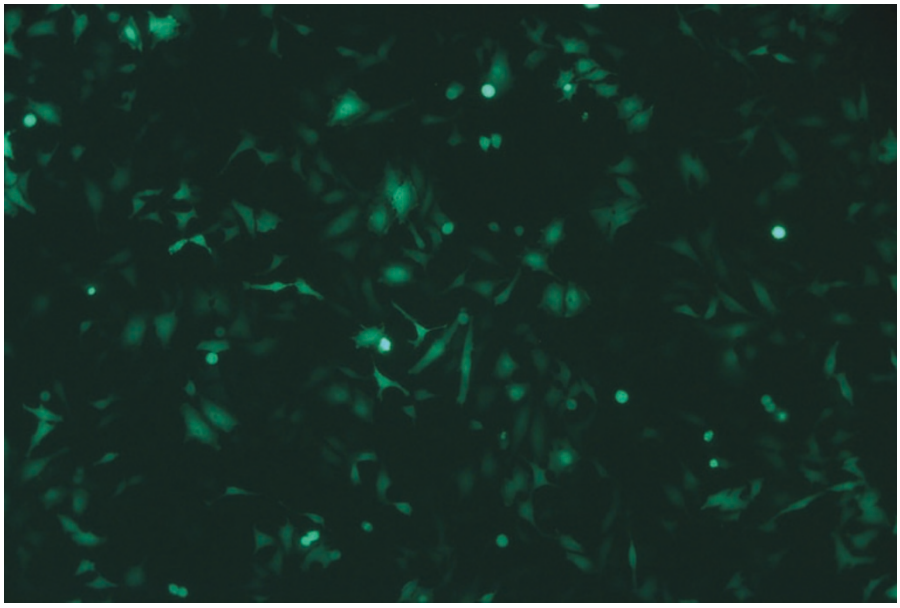
The inside scoop—evaluating gene delivery methods

Techniques for delivering nucleic acids into mammalian cells have been around for decades. But tools and reagents continue to improve and target a broader range of cells and applications. Laura Bonetta reports.

Pick a paper, any paper. Chances are that somewhere in the Methods section there is a description of a step for introducing DNA or RNA in cells. Although it has become a routine procedure, *in vitro* gene delivery can still be a challenge, especially when working with frail or scarce mammalian cells.

The mammalian cell membrane is impenetrable to large molecules that, like DNA, carry an electrical charge. Researchers have thus come up with an arsenal of tricks—from using carrier molecules and viral vectors to pocking holes in the membrane—to sneak nucleic acids through. But no one method can be applied to all types of cells or experiments. "You look at the articles that have been published and find one that has done some comparison of different transfection methods for your cells. But if you don't have any clues, you have to try different reagents, to find what will work best in your hands," says Nina Iversen, a researcher at the University of Oslo. The good news is that there is no shortage of reagents to try!



HeLa cells transduced with a self-inactivating retrovirus from Clontech's pQX (retroQ) series, which expresses green fluorescent protein. (Courtesy of Clontech.)

Solution revolution

A popular way to get DNA and RNA inside cells is to use carrier molecules. Such methods do not require expensive equipment and, from a technical standpoint, are less difficult to use than viruses. They rely on the fact that nucleic acids can interact with positively charged molecules, such as cationic lipids and polymers, to form complexes that are more palatable to the cell.

Carrier molecules on the market today owe their existence to the discovery, almost four decades ago, that coating DNA with DEAE-dextran would allow it to get past the cell membrane[1]—a process dubbed 'transfection'. In the early 1970s, researchers discovered that they could also transfect DNA using calcium phosphate, a less toxic chemical[2]. Some labs still use these reagents, which can be purchased as stand-alones or in kits. For example, Promega's ProFection mammalian transfection systems offer optimized buffers and solutions for either calcium phosphate– or DEAE-dextran–mediated transfection. But for many researchers, the newer, lipid-based reagents offer greater ease of use and efficiency with lower toxicity.

The first lipid-like molecules to come on the scene were mixtures of cationic and other lipids that would form artificial liposomes. Developed in the late 1980s, liposomal transfection reagents work by enveloping the DNA or RNA and then fusing with the cell membrane to deposit the nucleic acid cargo inside. Roche Applied Science sells liposome formulations based on the cationic lipids DOTAP and DOSPER. Qbiogene's MegaFectin combines DOTAP with different lipids. Under optimized conditions, liposome-mediated methods yield high efficiencies and are much easier to use than calcium phosphate.

Of lipids and polymers

The latest generation of lipid-based transfection products includes multicomponent, nonliposomal reagents consisting of lipids, polymers and combinations thereof. Although their composition is proprietary, most of them work by forming a complex with DNA or RNA that interacts with the cell membrane. The complex is believed to be taken up by endocytosis and then released in the cytoplasm. "Unlike the liposomal agents which form spheres that vary greatly in size, nonliposomal lipids

form micelles of uniform size resulting in more reproducible results," says Jamuna Ramnath, technical service scientist at QIAGEN Inc.

In the lipid arena researchers have a wealth of reagents to choose from. Invitrogen's primary product is Lipofectamine 2000, which works with a variety of nucleic acids and cell lines. It is also applicable to high-throughput screens. "It is quite stable after it makes a complex with DNA, so samples can sit on the deck of a robot loader for a long time," says Henry Chiou, manager for R&D at Invitrogen Corporation (**Box 1**).

Roche Applied Sciences' premier transfection reagent is the FuGENE 6 Transfection Reagent, a nonliposomal formulation of proprietary compounds, which has been shown to successfully transfect more than 700 different cell lines. "One of the advantages of FuGENE 6 over other transfection reagents is its broad applicability. It can be used with a wide range of cell lines without optimization and is effective in serum-containing media allowing for a simple consistent method" says product manager, Jeffrey Emch. The reagent also seems to minimize the chance of nonspecific, off-target effects—in other words, effects that are not caused by the transfected DNA or RNA. "We have done studies comparing up- and downregulation of off-target gene expression, and we feel comfortable that FuGENE 6 limits off-target effects," says Emch. "In an internal study, off-target effects by FuGENE 6 were dramatically less than a leading competitive agent."

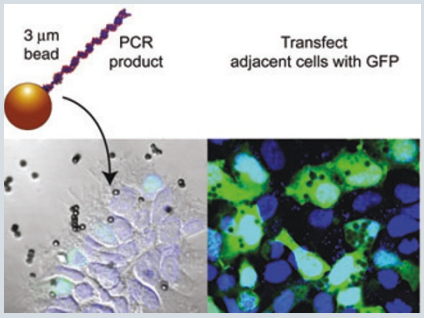
In addition to buffering the negative charge on the DNA many reagents also make the cargo smaller to facilitate delivery into cells. InvivoGen's LipoGen combines a lipid that fuses with the cell membrane with spermine, a divalent cationic polyamine that interacts with DNA and condenses it into compact lipid-DNA complexes. Similarly, QIAGEN's Effectene transfection reagent consists of a small positively charged molecule (called 'condensing enhancer') that shrinks the DNA in a dense structure and a nonliposomal lipid that coats the DNA allowing it to interact with the cell surface. Effectene works in the presence of serum and shows low cytotoxicity.

Another line of products that compact DNA includes QIAGEN's SuperFect and PolyFect reagents. They consist of activated spherical molecules (7 nm in diameter) with positively charged tentacles. The DNA coils around these molecules into histone-like structures that can enter the cell by endocytosis. "The dendrimer molecules ensure reproducibility from one experiment to the next," says Ramnath.

Once they have been taken up by endosomes, the DNA-carrier complexes must escape into the cytosol before being degraded (**Box 2**). Some carrier molecules are designed to help this process along. Metafectene, distributed by Biontex, is a polycationic transfection reagent based on what the company calls "repulsive membrane acidolysis" technology. When carrier and DNA are taken up in the endosome, the acidic environment induces the

BOX 1 GOING THE HIGH-THROUGHPUT WAY

Say you need to screen hundreds of cDNAs to search for genes that, when overexpressed, induce cell death. Or maybe you want to knock down several genes believed to function in the same pathway to better delineate the steps involved. The traditional way to transfect many cells at once is to place the cells in 96- or 384-well plates, add the DNA (or RNA) and transfection reagents, and then study the cells. Alternatively, the plates containing cells and DNA are loaded in a high-throughput electroporation instrument and processed. Given the amount of work involved, these protocols typically use robots to dispense the reagents and cells, as well as automated plate readers or microscopes to analyze the results.



Magnetic beads coated with DNA encoding green fluorescent protein are used to direct the transfection of adjacent HEK293 cells. (Courtesy of Mark Isalan.)

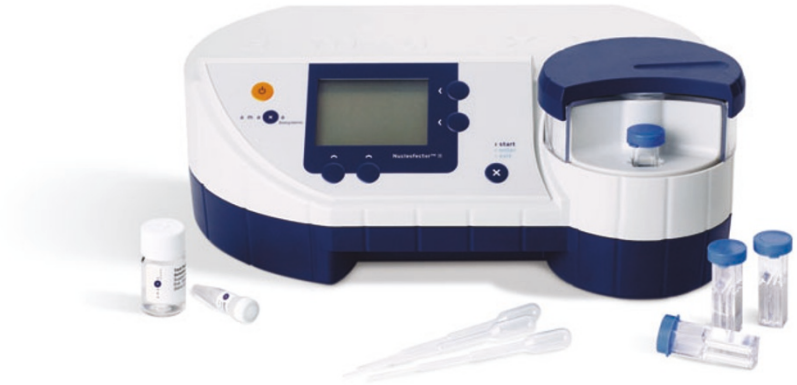
In an effort to simplify high-throughput transfection protocols Ziauddin and Sabatini[3] developed a new method based on microarray technology. In a transfection microarray, plasmid DNA dissolved in a gelatin solution is printed on a glass slide and then covered with a lipid-based transfection reagent. After removing the excess reagent, the slide is placed in a culture dish and covered with cells in medium. Cells growing on the printed areas take up the DNA creating spots of localized transfection within a lawn of nontransfected cells. (In an alternative version of this method the lipid-based transfection reagent is added to the DNA prior to printing.) Because cells are added to the reagent, this approach was called 'reverse transfection'.

A recent study describes another procedure for conducting parallel cell transfections on microscope coverslip arrays, but using a 'forward' methodology[4]. "We achieved transfection of a variety of cell lines using magnetic beads coated with PCR products," says Mark Isalan of the European Molecular Biology Laboratory and first author of the study. According to the protocol, cells are grown on a glass coverslip or slide to which magnetic microbeads coated with DNA are added. Using magnets, the beads are directed to the surface of individual cells where transfection occurs. "We can control the position of the beads to determine which cells to transfect," explains Isalan. "We can deliver DNA with micrometer resolution." The efficacy of the technique, which also works with 96-well plates, is greatly enhanced by adding a transfection reagent to the mix.

Isalan *et al.*[5] used this technology to engineer an artificial version of a gene network involved in embryonic patterning in the fruit fly. The scientists created a synthetic embryo by using a tiny plastic chamber containing various purified genes, proteins, metabolites and cell extracts. Some of the genes were attached to magnetic microbeads, so that they could be directed to specific locations using magnets anchored to the bottom of the chamber, to form a gene expression network.

carrier to separate from its cargo. Repulsive forces among the positively charged lipophilic parts of the lipids then ease the disruption of the endosomal membrane and, thereby, the release of the genetic material. According to the manufacturers, Megafectene is suitable for high-throughput applications by ensuring high transfection efficiency with small amounts of DNA and reagent.

Cationic polymers, such as poly(lysine) and poly(ethyleneimine) (PEI), also target release from the endosome. Fermentas' ExGen 500 reagent interacts with DNA to form small, stable, highly diffusible complexes, which are readily endocytosed. The "proton-sponge" effect of ExGen 500 buffers endosomal pH by provoking massive proton accumulation and passive chloride influx. Rapid osmotic swelling causes endosomal rupture, allowing translocation of DNA to the nucleus without DNA degradation. "An important feature is that it works with both primary and secondary cells. There is little to no cytotoxicity," says Matthew Crouch, product specialist for Fermentas Inc. "It is also cheaper than lipid-based reagents." Sigma-Aldrich also provides a PEI-based reagent called ESCORT V. Qbiogene has gone a step further by providing PEI-based reagents that couple the carrier molecule to ligands for specific receptors to make it easier to target specific cell types.



Amaxa's Nucleofector. (Courtesy of Amaxa.).

Despite the large number of available products, companies are continuing to perfect their blends to produce reagents that can be applied to as broad a spectrum of cells and applications as possible. In particular, many have optimized their transfection reagents to deliver short pieces of RNA to the cytoplasm to silence gene

expression (**Box 3**). Another focus for most companies is to develop better reagents for disease-relevant cells—such as stem cells and human primary cells—which are difficult to transfect. "It is still not a science where can look at a structure and predict what will work best. A lot of the work we do is empirical and requires setting up a variety of screens," says Invitrogen's Chiou.

Exploiting mother nature

Transfection using lipid- or polymer-based reagents is sometimes referred to as a chemical method, although no chemical reactions are involved, to distinguish it from approaches that use more 'natural' carriers. Nucleic acids can be introduced in cells by hitching a ride on a virus—an approach called transduction. "It is the most efficient system. The virus naturally evolved to get inside cells so it is readily possible to get 90–100% transduction efficiency of primary cells," says Andrew Farmer, director of cell and molecular biology at Clontech.

A typical transduction protocol involves engineering a viral vector to carry a gene of interest. The vector is then introduced into a packaging cell line to produce recombinant viral particles. The particles are then collected, sometimes purified and titered depending on the application, and used to infect the cells of interest.

The downside of working with viruses is that they present biosafety issues. In addition, transduction protocols may require arduous and time-consuming preparations of vectors and recombinant viruses. New products on the market, however, facilitate the process by providing complete kits that include vector and reporter plasmids, packaging cells, PCR primers as well as all the necessary buffers and solutions.

Clontech sells a broad range of systems for DNA transduction using retroviral and adenoviral vectors, each with its own advantages and disadvantages. Retroviruses insert into the genome of the cell that is transduced and are therefore useful for making stable cell lines, but they can only infect diving cells. Adenoviruses, on the other hand, do not integrate into the genome but can transduce a broad spectrum of cell types with high efficiency. (These two viruses make up the major portion of the viral-vector market.)

In addition to providing complete transduction kits, Clontech sells an adenovirus purification kit. "It is a column that allows you to take the packaging cell lysate and pass it over a filter to produce purified, concentrated virus in about three hours," says Farmer. The company is developing a similar purification kit for retrovirus. Clontech also sells titration kits that make the determination of the concentrations of infectious virus much easier than with traditional plaque assays.

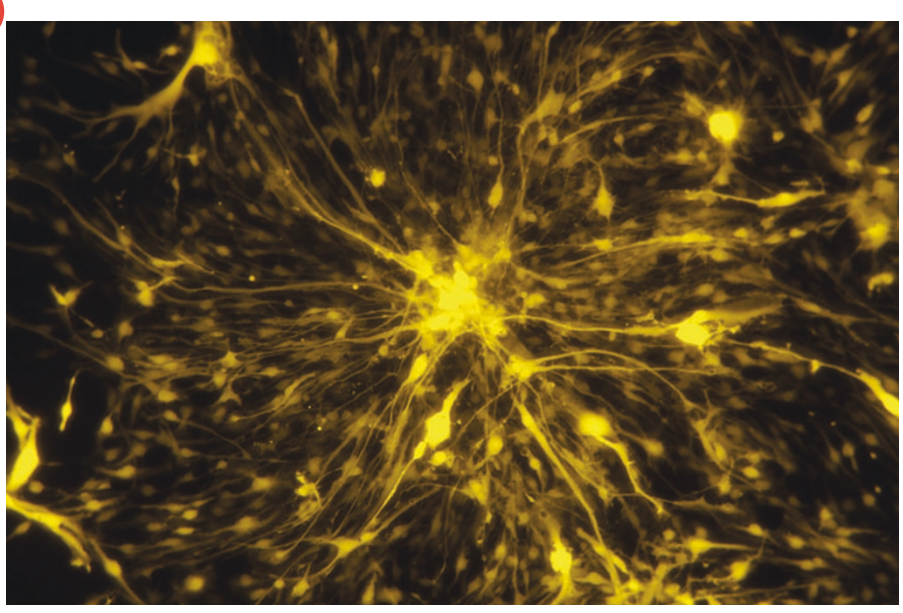
Lentiviruses, a subclass of retroviruses that includes HIV, are relative newcomers to the viral vector market. Like all retroviruses, they can integrate DNA into host cells, but can infect cells that are not dividing. "They can go into a wider range of cells and create stable cell lines," says Chiou. "They are continuing to gain popularity." Invitrogen sells both lentivirus- and adenovirus-based transduction kits.

Adeno-associated virus (AAV)-based systems have also been gaining ground in recent years, in part thanks to kits such as Stratagene's AAV helper-free system. Recombinant AAV are capable of transducing a broad range of both dividing and nondividing cell types, and are less toxic than adenovirus. The major downside of using AAV, however, is that the virus needs to be coinfected with adenovirus to produce recombinant AAV virions in pack-

BOX 2 TARGET: THE ENDOSOME

Most macromolecules enter mammalian cells by endocytosis. But a large proportion of molecules taken up in this way will become trapped inside endocytic vesicles and be degraded before they are able to exert a biological effect. For this reason, many carrier molecules used in transfection protocols are designed to maximize the release of the carrier-DNA complex into the cytosol. A method called photochemical internalization, which targets the endosome, claims to improve gene delivery by most synthetic and viral vectors even further[6].

The method uses photosensitizing compounds that are taken up by cells and localize specifically to the membranes of endosomes. When cells are exposed to light, the photosensitizers cause endosomes to rupture. "Approximately fifty percent of everything that was taken up by endocytosis is released," says Kristian Berg of the Norwegian Radium Hospital in Oslo, who pioneered the technique. "It is a general method to be used for all types of molecules intended to enter the cytosol or nucleus." The Norway-based company PCI Biotech sells the light source (LumiSource) and photosensitizing reagents (LumiTrans).



A colony of differentiating primary human neural progenitor cells transduced with an adenovirus expressing the DsRed Fluorescent protein. (Courtesy of Clontech.)

aging cells. Stratagene's vector system eliminates the need for the helper virus, greatly simplifying the transduction procedure.

Bringing in the guns

When all else fails, electroporation is the brute force approach for delivering genetic material inside cells. Cells suspended in a solution are bombarded with high-voltage pulses of electricity that produce transient holes in the cell membrane, through which the DNA can enter. Electroporation is a harsh technique and many cells will die in the process. For this reason, researchers typically use greater quantities of DNA and cells than with other methods. On the other hand, the technique can be used with almost all types of cells with some success.

Many companies sell electroporators, cuvettes and buffers. Popular instruments include Bio-Rad's Gene Pulser Xcell system and BTX Molecular Delivery Systems' ECM line of electroporators. Recently BTX has released a new product for high-throughput transfections. The BTX HT 96-well electroporation system has a plate handler with auto-track sensing, which allows rapid electroporation of an entire plate using different electrical parameters for all columns, at the press of a button.

A welcome twist to the electroporation technique comes from Amaxa. The Nucleofector technology is similar, in principle, to electroporation, but the nucleic acids are delivered directly to the cell's nucleus. The technology is suitable for both DNA and RNA and provides greater viability of transfected cells. "One advantage is that gene expression starts earlier," says Liz Horton at Amaxa. "It works with primary cells that are typically difficult to transfect by other methods." Amaxa has over 40 optimized kits and protocols specifically designed for different primary cells, along with five kits that can be optimized for use with the most popular primary mammalian cell types. In addition, Amaxa has optimized protocols for over 50 common cell lines, which use one of five general cell line kits. To assist in the optimization process, the company's website contains a database that lists customers' data.

Several studies have shown that nucleofection is effective in transfecting stem cells, neuronal cells and other primary cells. "Nucleofection worked well in our hands," says Uma Lakshmipathy at the University of Minnesota Medical School, who used Nucleofector technology to introduce genes into both embryonic and adult stem cells to induce them to

The Helios gene gun. (Courtesy of Bio-Rad.)

differentiate into different lineages. Iversen agrees. "Our experience is that it works very well. The only problem is that you need a lot of cells," she says. Amaxa is now developing a new 96 well–plate system for high-throughput experiments, which uses small volumes with low cell numbers. "You won't have to have the same parameters for each well," says Horton. The system allows for each well to be handled independently, using up to 96 different conditions.

In addition to electroporation, several other physical methods to get DNA inside cells exist. About ten years ago Bio-Rad developed an alternative to electroporation with the Helios gene gun, a hand-held device that uses an adjustable low-pressure helium pulse to shoot tiny gold pellets coated with DNA or RNA into cells in tissues. "In electroporation the current causes the membrane to open and an electophoretic effect lets nucleic acid drift in. But the 'biolistic' approach forces the particles in," says Steve Kulisch, product manager of the gene expression division at Bio-Rad Laboratories. In particular, Bio-Rad's gun has found a niche in genetic vaccination and agriculture applications. A benchtop version of the technology can be used for routine transfections in research labs.

Another physical transfection approach is Magnetofection, a new method that associates nucleic acids or transfection

BOX 3 RNAi CRAZE

RNA interference (RNAi)—a method for knocking down the expression of specific genes—has taken molecular biology by storm. The approach works by introducing (21–23 nucleotide) siRNAs in mammalian cells, either directly or via a plasmid or virus, prompting the cleavage of endogenous mRNA homologous to the particular siRNA.

Several reagents, such as Invitrogen's Lipofectamine 2000, were originally developed for plasmid DNA but also work well with siRNAs. In many cases, however, companies have produced additional reagents specifically targeted to the siRNA market. QIAGEN offers two: RNAiFect, based on a nonliposomal lipid formulation, and HiPerfect, a unique blend of cationic and neutral lipids that allows effective siRNA uptake and silencing of gene expression using low concentrations of siRNA. Transfection with HiPerfect can be performed in the presence of serum without removing the complexes. "It can be applied to high-throughput technology," says Ramnath. Roche's X-tremeGENE siRNA Transfection Reagent efficiently delivers different siRNAs into a wide range of eukaryotic cell lines with high transfection and knockdown efficiency. It is also effective for cotransfecting siRNA and plasmid DNA.

Reagents for siRNA delivery include Upstate's siIMPORTER, Bio-Rad's siLentFect and Novagen's RiboJuice (a formulation that combines amine- and lipid-based reagents), among others. Dharmacon, one of the leading providers of RNAi technologies, recently announced the introduction of a new set of siRNA transfection reagents—Dharma*FECT 1*, 2, 3 and 4—which are available either individually or as a set. Dharma*FECT* 1 works across the broadest range of cell types. Dharma*FECT* 2 has the least toxicity. Dharma*FECT* 3 delivers siRNA efficiently in certain cell lines such as LNCaP, and Dharma*FECT* 4 works well for mouse and rat cell lines.

One of the problems of using cationic carriers to deliver siRNA inside cells is the production of side effects that lead to nonspecific changes in the expression profiles of several genes—or, in other words, off-target effects. According to some manufacturers, the newer products minimize the problem. Protocol optimization also helps to take care of the problem. "You have to adjust the amount of lipid you use to reduce or eliminate off target effects. Researchers should determine the minimum amount of carrier that is required," says Chiou.

siRNAs can also be transfected using electroporation or other physical methods. Ambion sells electroporation buffers specialized for siRNA. The product is sold on its own and as part of the siPORT electroporation kit, which also contains several transfection controls.

reagents or viruses with cationic magnetic nanoparticles and exploits magnetic force to transport nucleic acids into cells (see also **Box 1**). According to Oz Biosciences, the company that markets this technology, the complete dose of applied vector is concentrated on the cells within a few minutes and transfection is very efficient for many cell lines including hard-to-transfect ones and primary cells. Oz Biosciences designed four different cationic nanoparticles formulations applied to specific applications: PolyMag can be used with all nucleic acids, including plasmid DNA, RNA, oligonucleotides and short interfering RNA (siRNA); SilenceMag is specifically designed for siRNA delivery; ViroMag is dedicated to viral applications; and CombiMag can be used in combination with transfection reagents to boost their efficacy. "The appeal of the Magnetofection technology is in its versatility toward nucleic acids types, transfection reagents and virus, its efficiency and universitality in cells successfully tested," says Olivier Zephati, CEO of Oz Biosciences.

When it comes to delivering nucleic acids inside cells, options abound. The advice most researchers give is to test several reagents or techniques by setting up a matrix of conditions. For this purpose, many companies sell optimization kits and trial-sized aliquots or even provide researchers with free samples to try. In addition, the websites of companies that supply transfection reagents often contain databases of transfection protocols for different cell types and applications.

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5. Isalan, M. *et al. PLoS Biol.* **3,** e104 (2005).
6. Berg, K. *et al. Cancer Res.* **59**,1180–1183 (1999).

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SUPPLIERS GUIDE: COMPANIES OFFERING GENE DELIVERY SOLUTIONS

Company	Web address
Amaxa	http://www.amaxa.com/
Ambion	http://www.ambion.com
Amersham Biosciences (part of GE Healthcare)	http://www4.amershambiosciences.com
Avanti Polar Lipids Inc.	http://www.avantilipids.com/
B-Bridge International Inc	http://www.b-bridge.com/eng/index.htm
Bender MedSystems	http://www.bendermedsystems.com/
Bio-Rad Laboratories	http://www.bio-rad.com
BTX Molecular Delivery Systems	http://www.btxonline.com/
Clontech (BD Biosciences)	http://www.clontech.com/clontech/
Dharmacon, Inc.	http://www.dharmacon.com/
Eppendorf	http://www.eppendorf.com
Eurogentech	http://usa.eurogentec.com/code/en/hp.asp
Fermentas	http://www.fermentas.com/
GeneChoice, Inc.	http://www.genechoiceinc.com/
Geneflow Ltd.	http://www.geneflow.co.uk
Genlatis	http://www.genlantis.com/
Imgenex	http://www.imgenex.com
Invitrogen	http://www.invitrogen.com/
InvivoGen	http://www.invivogen.com/
Midwest Scientific	http://midsci.com/
Microbix	http://www.microbix.com/products/04.html
Mirus	http://www.mirusbio.com/
MoBiTec Molecular Biotechnologies	http://www.mobitec.de/mobitec_us/index.html
Molecula	http://www.molecula.com/
MP Biology	http://www.mpbio.com
Oz Biosciences	http://www.ozbiosciences.com/English/default.html
Polyplus Transfection	http://www.polyplus-transfection.com/EN/index.php
Pepscan Systems	http://www.pepscan.nl
PCI Biotech	http://www.pcibiotech.com/
PGC Scientific	http://www.pgcsci.com
Promega	http://www.promega.com/
Qbiogene Inc.	http://www.adenovirus.com/
Qiagen	http://www1.qiagen.com/
Roche Applied Science	http://www.roche-applied-science.com/
Sigma-Aldrich	http://www.sigmaaldrich.com/
Stratagene	http://www.stratagene.com/homepage/
System Biosciences	http://www.systembio.com
Targeting Systems	http://www.targetingsystems.com/
Thermo Electron Corporation	http://www.thermo.com
Tritech Research Inc.	http://www.tritechresearch.com/cgi-bin/shop/
Upstate	http://www.upstate.com
Wako Chemicals SA	http://www.wakousa.com/