Delitto Per

Christopher G. Reuther/EHP

Foreign DNA Disappears without a Trace

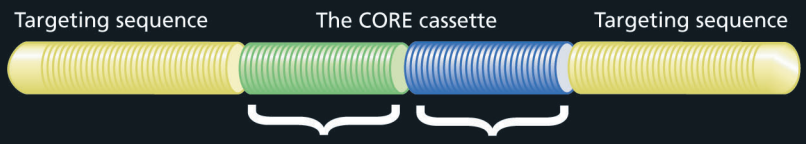
A group of NIEHS scientists has recently perfected a new *in vivo* gene modification technique that enables researchers to quickly generate site-directed mutations onto specific regions of the yeast genome without leaving behind foreign DNA. The new technique is transforming the way genetic researchers analyze how genes function and respond to human disease and environmental influences.

The research team is composed of Michael Resnick, head of the institute's Chromosome Stability Group, postdoctoral fellow Francesca Storici, and former group member L. Kevin Lewis, now an assistant professor of biochemistry at Southwest Texas State University in San Marcos. With encouragement from her colleagues, Storici—who hails from Trieste, Italy, where she conducted research at the International Centre for Genetic Engineering and Biotechnology—christened the new technique "Delitto Perfetto," a Italian term that means *perfect deletion* and is also idiomatic for *perfect murder*. Just as the perpetrator of the perfect murder leaves no clues behind, this novel gene modification technique leaves no trace of the foreign DNA first introduced to engineer the desired genetic changes.

The Delitto Perfetto Process

The CORE cassette consists of two marker genes. When these genes become incorporated into a yeast cell, they determine on which types of media the cell can grow. This is vital for identifying cells that have been successfully manipulated.

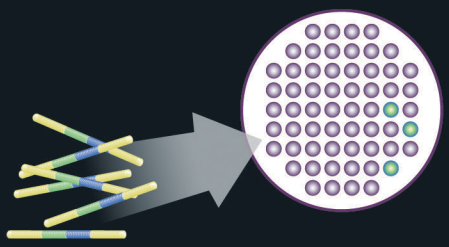
On either side of the marker genes are DNA sequences that target the cassette to the particular spot in the genome where a change is desired.



Conterselectable (CO) marker gene: Cells that express the CO marker gene can be directly selected by their ability to grow on medium A, while cells that have lost this gene can be selected by their ability to grow on medium B.

Reporter (RE) marker gene: Cells that express the RE marker gene can be directly selected by their ability to grow on medium C.

CORE cassettes are added to yeast cells.



Through the process of recombination, prior to cell division, a few yeast cells will incorporate the CORE cassette into their genomes at the point determined by the targeting sequences. This is the point at which new DNA will be inserted later.

Modus Operandi

Scientists rely on several methods to study human genes. One involves isolating a human clone within a model organism and studying it. (In 1996 Resnick, with NIEHS colleagues Vladimir Larionov and Natalya Kouprina, perfected a method using yeast artificial chromosomes, or YACs, for the specific isolation of entire human genes.) In another, changes are introduced into homologous genes or cloned genes in model systems. Scientists can make these changes by introducing vector DNA that does not correspond to the host organism's DNA. Resnick says the possibility of foreign DNA being expressed in the genome of a genetically altered organism is one of the concerns of genetic engineering.

"Delitto Perfetto provides a new dimension to YAC cloning of human DNA," Resnick says, "because it gives one the opportunity to modify genes directly on the YAC without any subcloning process. Until now, targeted recombination and modification has been a rather slow process."

"The idea [for Delitto Perfetto] emerged from a combination of approaches, including a DNA cassette cocktail," Storici explains. The cocktail includes two gene markers, one a **CO**unterselectable gene whose absence can be selected for, one a **RE**porter whose presence can be selected for. Together, the markers form a so-called **CORE** cassette within a region of DNA 3,200 base pairs long.

Delitto Perfetto is a two-step, cloning-free method: First, the CORE cassette containing genetic markers is targeted into yeast DNA, to a particular DNA sequence where the mutation, deletion, or insertion is desired. Next, the cells containing the CORE cassette are transformed with oligonucleotides that are designed to contain the chosen modification, a process that yields the desired deletion, insertion, or mutation.

The oligonucleotide transformation process leads to the loss of the CORE cassette that contained the foreign DNA. Once the mutagenesis is complete, only the genetic material showing the desired change remains. The reporter marker monitors the insertion of the cassette, and the counterselectable marker monitors loss of DNA during mutagenesis.

A second approach involves exploiting the highly efficient homologous recombination system of the yeast *Saccharomyces cerevisiae*, where gene targeting is possible using very short homologous regions. The group showed that unpurified commercially available oligonucleotides could be used, greatly simplifying the site-directed mutation process.

Delitto Perfetto is a very flexible system. "We can put the CORE cassette anywhere we want in the genome," says Resnick. Once the cassette is inserted in a certain location, many modifications can be introduced by transforming with different oligonucleotides. "Using oligonucleotides of fifty to one hundred base pairs, we target those for the region surrounding the inserted CORE cassette," he says. "In the process, we delete the cassette and are left with the sequence of oligonucleotides that matches the original gene, except for the change that was introduced."

Using Delitto Perfetto, Resnick's team has so far produced single- and multiple-point mutations, short deletions and insertions, and extensive deletions of precise nucleic acid sequences. They have also examined a variety of individual and complementary oligonucleotides, along with parameters that influence site-directed mutagenesis, including oligonucleotide length.

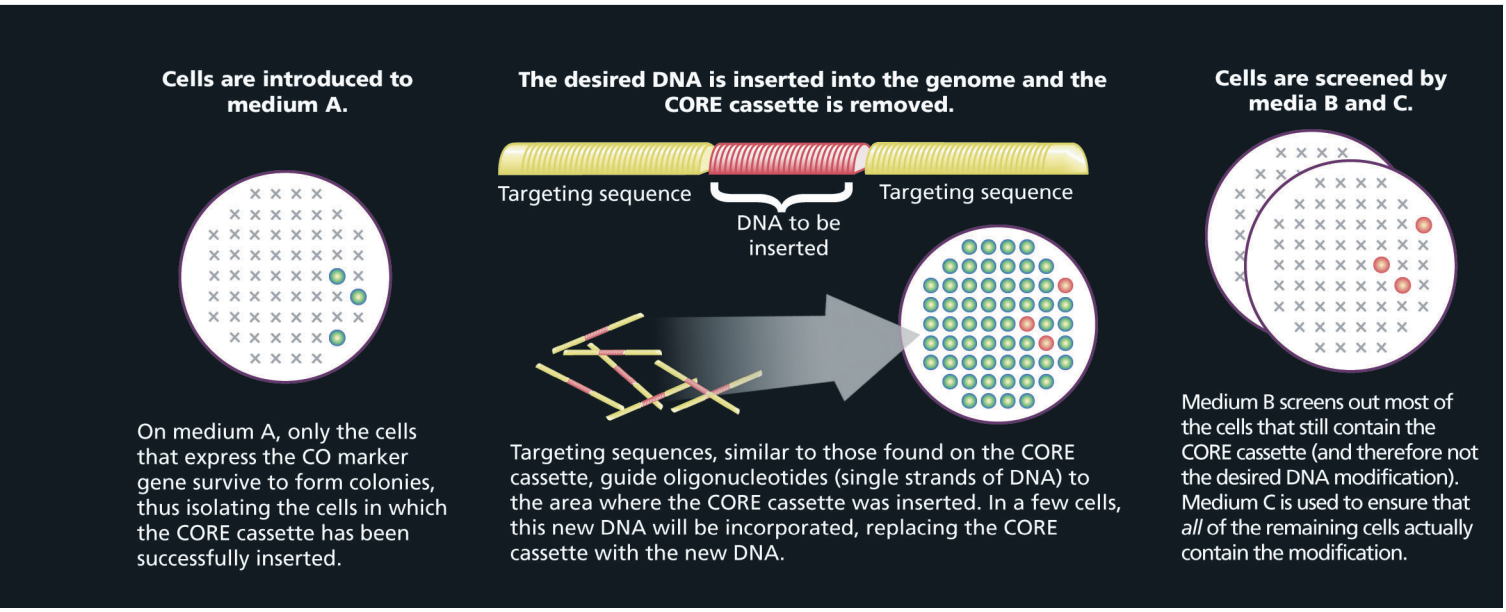
The technique's versatility makes it ideal for producing multiple rounds of specific or random changes within a specific range of up to 200 base pairs. Delitto Perfetto also provides opportunities to study basic mechanisms of mutation and recombination that contribute to genome instability.

The Weapon of Choice

Yeast, as it turns out, is the magic medium for the team's studies. Fred Sherman, a professor in the Department of Biochemistry and Biophysics at the University of Rochester Medical School in New York, who has followed the work of Resnick's team, says many important protein genes have their counterpart in yeast, making it quite useful for structural studies of proteins. As *S. cerevisiae* has the best-characterized eukaryotic genome, it has proven itself ideal for many cross-species studies. Thus, it allows researchers to experimentally manipulate large heterologous genomic DNA fragments, cloning them into YACs. Previous systems have allowed scientists to modify natural chromosomes or YACs without leaving a trace of any heterologous sequence, but those methods offer limited flexibility and are extremely laborious and time-consuming.

In contrast, Delitto Perfetto is fast. Scientists typically study yeast cells by creating different versions of proteins, which, when done "by hand," is painstakingly slow and laborious. After the cassette is put into place using the Delitto Perfetto technique, researchers can study many different gene alterations quickly and cost-effectively. "Once [the cassette] is inserted in position, it is very fast—you don't have to repeat the process," Storici says. The two steps, she says, take a total of 12 days to complete.

Christopher G. Reuther/EHP, Francesca Storici



"Once [the cassette] is created," Resnick adds, "you have the opportunity to do any number of changes in the region—hundreds of specific changes and many random changes within a week." He says the technique is accurate about 80% of the time, which is as efficient and accurate as standard *in vivo* mutagenesis systems, and the accuracy can be checked relatively easily.

One disadvantage, says Sue Jinks-Robertson, a professor in the Department of Biology at Emory University in Atlanta, is that Delitto Perfetto limits site-directed mutagenesis to a small region of the genome surrounding each inserted cassette, a limitation balanced by the fact that researchers usually want to confine mutagenesis to one functionally important domain anyway. Another drawback could be the cost of commercially available oligonucleotides. However, Jinks-Robertson says, "This cost should be more than offset by the time saved."

Jinks-Robertson agrees that the technique shows promise, especially when compared with traditional methods, which typically involve performing the mutagenesis of the gene of interest on a plasmid, followed by a standard two-step gene replacement method. "Not only is this a lengthy procedure for each desired mutation, but the mutagenesis process itself—usually a polymerase chain reaction—often introduces unwanted additional mutations," she explains. "With the Delitto Perfetto method, oligonucleotides containing the desired mutation are directly transformed into yeast, where they recombine directly with the genomic target."

Says Sherman, "What Resnick and his colleagues have done is develop a method to make changes more conveniently. It's especially useful for someone doing a systematic study requiring numerous replacements and deletions. If one were to make many changes to many amino acid sequences, it would be very helpful." Sherman considers Delitto Perfetto an important addition to functional genomics.

Resnick believes the technique will make "a major impact [because] it will allow rapid access to gene functions and lead to better understanding of the gene functions that cause environmental responses." For example, Delitto Perfetto could allow scientists to rapidly study repair genes and possibly lead to better model systems for identifying drugs. "We can rapidly create [gene] modifications that correspond to polymorphisms, then study how they respond to environmental agents," Resnick explains.

The team has begun to analyze the recombination proficiency of oligonucleotides with different secondary structures to pinpoint substrates that will render the technique even more efficient. "Experiments are also under way to test the efficacy of oligonucleotide-directed recombination and mutagenesis within mammalian cells," Resnick says.

The team has also used the technique to analyze functions of the *p53* gene (a tumor suppressor that is the most commonly mutated gene in human cancers); to study the human *FEN1* gene (important in DNA replication and repair); and to evaluate the stability of DNA sequences from the human genome. "We plan to utilize the approach to generate random and specific substitutions in functional domains of human *FEN1* and *p53*," Resnick says. The team also plans to engineer specific modifications of human genes cloned as large genomic DNAs in yeast (as YACs), which they hope to transfer directly into human cells so they can study the functions of altered genes in the human cell.

The technique may also apply to other organisms where homologous recombination is efficient, including the moss *Physcomitrella patens* and DT40 chicken cells (used to study human chromosomes). Delitto Perfetto could also be used to modify large bacterial artificial chromosomes using recently developed strains of *Escherichia coli* that have proven efficient for site-specific targeting. Because yeast is routinely used for random and selective cloning of genomic DNA from higher eukaryotes (in the form of YACS), Resnick contends, this strategy provides an efficient method for creating precise changes in mammalian or other heterologous DNA sequences.

Jennifer Medlin

Suggested Reading

Erdeniz N, Mortensen UH, Rothstein R. 1997. Cloning-free PCR-based allele replacement methods. Genome Res 7:1174–1183.

Kmiec EB. 1999. Targeted gene repair. Gene Ther 6:1–3.

Muyrers JP, Zhang Y, Benes V, Testa G, Ansorge W, Stewart AF. 2000. Point mutation of bacterial artificial chromosomes by ET recombination. EMBO Rep 1(3):239–243.

Storici F, Lewis LK, Resnick MA. 2001. *In vivo* site-directed mutagenesis using oligonucleotides. Nat Biotechnol 19(8):773–776.