

Science Education Collection

Recombineering and Gene Targeting

URL: <https://www.jove.com/science-education/5553>

Abstract

One of the most widely used tools in modern biology is molecular cloning with restriction enzymes, which create compatible ends between DNA fragments that allow them to be joined together. However, this technique has certain restrictions that limit its applicability for large or complex DNA construct generation. A newer technique that addresses some of these shortcomings is recombineering, which modifies DNA using homologous recombination (HR), the exchange between different DNA molecules based on stretches of similar or identical sequences. Together with gene targeting, which takes advantage of endogenous HR to alter an organism's genome at a specific loci, HR-based cloning techniques have greatly improved the speed and efficacy of high-throughput genetic engineering.

In this video, we introduce the principles of HR, as well as the basic components required to perform a recombineering experiment, including recombination-competent organisms and genomic libraries such as bacterial artificial chromosomes (BAC). We then walk through a protocol that uses recombineering to generate a gene-targeting vector that can ultimately be transfected into embryonic stem cells to generate a transgenic animal. Finally, several applications that highlight the utility and variety of recombineering techniques will be presented.

Transcript

Cloning by homologous recombination, or recombineering, has greatly improved researchers' ability to conduct high-throughput genetic engineering. Classical molecular cloning requires digestion of vectors and inserts with the same restriction enzymes to generate compatible ends for "recombination," but especially when trying to isolate a region from a longer sequence, such as a stretch of genomic DNA, there are not always restriction enzymes that will cut uniquely around the region of interest, and not within the region or elsewhere in the sequence. By avoiding the need for these restriction sites, recombineering provides a much more efficient and cost-effective way to make genetic engineering constructs.

In this video, we will introduce the principles of genetic recombination, provide a general protocol for recombineering a gene-targeting vector, and discuss several applications of these technologies.

First, let's discuss what recombination is and how it can be applied.

Genetic recombination entails the exchange of information between DNA molecules. In "homologous recombination," relatively large stretches of similar or identical DNA sequences are exchanged. Cells use this process to repair double strand breaks, and sexually reproducing eukaryotes also carry this out between homologous chromosomes during meiosis to increase genetic diversity.

Homologous recombination has been harnessed for a number of experimental purposes, including the modification of specific endogenous loci in cells—called "gene targeting." It is also applied to the engineering of genetic constructs, a process called "recombineering," which is especially useful for making modifications to large stretches of DNA for targeting to an organism's genome. To do this, researchers can make use of "genomic libraries," or sets of vectors each containing long genomic DNA fragments. Various types of libraries with different capacities, usually propagated in bacterial clones, have been created and can be ordered from commercial or nonprofit repositories. One of the most commonly used types of genomic library is called a bacterial artificial chromosome or BAC, which can carry an insert size between 150-350 kilobases.

In order to recombineer a construct, scientists need an organism in which homologous recombination occurs. The yeast *Saccharomyces cerevisiae* is naturally "recombination-competent" and can readily carry out homologous recombination between a vector and insert. Another commonly applied system uses the "Red" proteins from bacteriophage lambda, which is reconstituted in the bacteria *E. coli*. Vectors can be recombineered either in previously established Red-expressing bacterial strains, or a plasmid encoding Red system components can be co-transformed with the recombineering substrates into the bacteria.

Now, let's walk through a protocol to create a gene-targeting vector by recombineering.

The aim of this protocol is to create a genetically modified mouse with targeted changes to a specific gene. Briefly, desired modifications to the gene of interest are made in a BAC; the modified sequence is "retrieved" into a targeting vector, and this vector is transfected into embryonic stem cells for gene targeting. The stem cells can then be used to generate the genetically modified animal. For instructions on this final procedure, please refer to the JoVE Science Education video "Genetic Engineering of Model Organisms."

To begin, a BAC clone containing the genomic sequence of interest is identified and ordered. Next, the homology arms, or oligonucleotides containing the 30-50 basepair homologous regions, are designed. These oligonucleotides are used as primers in PCR reactions to generate the DNA "cassettes" used to modify the gene of interest. These cassettes typically contain, for example, antibiotic resistance genes that can act as selection markers, which can be readily amplified from many publicly available plasmids. The BAC bacterial clone is then transformed, for example

by electroporation, with a plasmid encoding the Red system components, followed by the homology arm-containing cassettes. The bacteria are then incubated at the appropriate temperature to induce recombination.

To recover the modified sequence from the BAC, a “retrieval” vector is linearized and amplified using primers containing homology arms that cover several kilobases around the site of modification. This vector backbone is transformed into the BAC bacterial clone, and recombineering is induced again. The vector will be “gap-repaired” by “retrieving” the appropriate sequence from the BAC. This results in a gene-targeting vector containing the modified sequence of interest, as well as regions that are homologous to genomic DNA surrounding the modified sequence. This vector can then be purified, linearized and introduced into embryonic stem cells where the endogenous homologous recombination machinery will target the modified sequence to the corresponding locus, thereby altering the host genome.

Let’s now look at some applications of recombination-based engineering techniques.

First, researchers can use recombineering to quickly and easily engineer the bacterial genome, for example, to study protein complexes. Here, a cassette encoding a sequential peptide affinity tag as well as a kanamycin resistance selection marker was designed to be recombineered into the 3¢ end of the gene of interest. This vector was then introduced into Red “recombination-competent” *E. coli*, and successful recombinants were isolated using selective media. The tagged protein complexes were biochemically purified using proteins that bind strongly to the tags, and analyzed by mass spectrometry to identify the interaction partners of the protein of interest.

Recombineering and gene targeting can also be used to modify the genome of parasitic organisms, such as the obligate intracellular parasite *Toxoplasma gondii*, which causes the disease toxoplasmosis. In this study, a targeting vector was recombineered in yeast, where genomic sequences flanking the gene of interest were placed around a selection marker. The vector was then linearized and electroporated into the parasites. Limiting dilution plating in selective media was used to isolate successful recombinants, in which the parasite’s homologous recombination machinery replaced the genomic sequence with the engineered sequence, thus deleting the gene of interest. PCR of the targeted region confirmed positive recombination events.

Finally, a procedure known as subcloning plus insertion, or SPI, has been devised in which gap repair and cassette insertion occur simultaneously, making the recombineering process simpler and more flexible. Critical for the success of this process is the proper design of the multiple homology arms, which, at up to 180 bp, are longer than in conventional recombineering so as to increase recombination efficiency. After incorporating the homology arms into the subcloning plasmid and insertion cassettes by PCR, they were transformed together into a Red-expressing BAC clone, and recombineering was induced. Successfully recombineered constructs were then identified by PCR or restriction digest analysis.

You’ve just watched JoVE’s video on recombineering. In this video, we have discussed the principles of recombination and how they can be used in genetic engineering, a protocol for a recombineering project, and finally several applications of these techniques. As always, thanks for watching!