Restriction Endonucleases: Molecular Cloning and Beyond

The sequence-specific DNA cleavage activity of restriction endonucleases (REases), combined with other enzymatic activities that amplify and ligate nucleic acids, have enabled modern molecular biology. After more than half a century of research and development, the applications of REases have evolved from the cloning of exogenous DNA and genome mapping to more sophisticated applications, such as the identification and mapping of epigenetic modifications and the high-throughput assembly of combinatorial libraries. Furthermore, the discovery and engineering of nicking endonucleases (NEases) has opened the door to techniques such as isothermal amplification of DNA among others. In this review, we will examine the major breakthroughs of REase research, applications of REases and NEases in various areas of biological research and novel technologies for assembling large DNA molecules.

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INTRODUCTION

In the 1950s, a phenomenon known as "host controlled/induced variation of bacterial viruses" was reported, in which bacteriophages isolated from one *E. coli* strain showed a decrease in their ability to reproduce in a different strain, but regained the ability in subsequent infection cycles (1,2). In 1965, Werner Arber's seminal paper established the theoretical framework of the restriction-modification system, functioning as bacterial defense against invading bacteriophage (3). The first REases discovered recognized specific DNA sequences, but cut at variable distances away from their recognition sequence (Type I) and, thus were of little use in DNA manipulation. Soon after, the discovery and purification of REases that recognized and cut at specific sites (Type II REases) allowed scientists to perform precise manipulations of DNA *in vitro*, such as the cloning of exogenous genes and creation of efficient cloning vectors. Now, more than 4,000 REases are known, recognizing more than 300 distinct sequences (for a full list, visit REBASE® at rebase.neb.com). With the advent of the Polymerase Chain Reaction (PCR), RT-PCR, and PCR-based mutagenesis methodologies, the traditional cloning workflow transformed biological research in the decades that followed.

ENGINEERING OF RESTRICTION ENZYMES

Traditionally, REases were purified from the native organism. The development of gene cloning vectors and selection methodologies enabled the cloning of REases. Cloning not only allowed the production of large quantities of highly purified enzymes, but also made the engineering of REases possible. Currently, > 250 of the restriction enzymes supplied by New England Biolabs (NEB) are recombinant proteins.

Engineering Improved Performance

Cleavage activity at non-cognate sites (i.e., star activity) had been observed and well-documented for some REases. Of those, some exhibit star activity under sub-optimal reaction conditions, while others have a very narrow range of enzyme units that completely digest a given amount of substrate without exhibiting star activity (4). Through intensive research, scientists at NEB began engineering restriction enzymes that exhibit minimal, if any, star activity with extended reaction times and at high enzyme concentrations. This research enabled the introduction of High Fidelity (HF™) REases that have improved performance under a wider range of reaction conditions (for more information, visit www.neb.com/HF).

Engineering New Sequence Specificities

Attempts to alter the sequence specificities of Type IIP REases have been largely unsuccessful, presumably because the sequence specificity determinant is structurally integrated with the active sites of Type IIP REases. MmeI, a Type IIG REase with both methyltransferase (MTase) and REase activities in the same polypeptide, recognizes the target sequence TCCRAC using the target recognition domain (TRD) within its MTase component. This represented an excellent opportunity to engineer altered sequence specificity into the REase. As an added advantage, the sharing of the TRD between the REase and MTase activities resulted in an equivalent change in MTase activity for any change in target sequence cleavage specificity, protecting the new target site from cleavage in recombinant host cells. Through bioinformatics analysis of homologous protein sequences, scientists at NEB identified the amino acid residues that recognized specific bases within the target sequences and created MmeI mutants with altered sequence specificities (5). Rational design of MmeI mutants and homologs unlocked the potential for the creation of REases with hundreds of new sequence specificities.

Figure 1. Nicking Enzyme Engineering
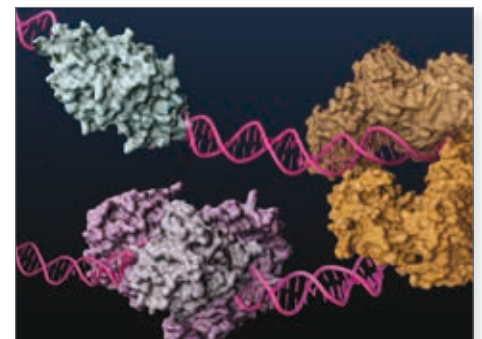
Type IIS REases, such as FokI (light and dark brown) and BstNBI (isoschizomer of BspD6I, light and dark purple), and homing endonuclease I-AniI (cyan), have been engineered to possess nicking enzyme activities.

TYPES AND ACTIVITIES OF RESTRICTION ENZYMES

Type I
Cleaves DNA at random sites far from its recognition sequence

Type II
Cleaves DNA at defined positions close to or within its recognition sequence

Type IIG
Cleaves outside its recognition sequence with both REase and MTase enzymatic activities in the same protein

Type IIP
Cleaves symmetric targets and cleavage sites

Type IIS
Recognizes asymmetric sequences

Type III
Cleaves outside its recognition sequence and require two sequences in opposite orientations within the same DNA

Type IV
Cleaves modified (e.g., methylated) DNA

Engineering Nicking Endonucleases

Basic research involving REases led to surprising findings about the seemingly straightforward mechanism of cleavage. Prototypical Type IIP REases normally act as homodimers, with each of the monomers nicking half of the palindromic site. Type IIS REases, on the other hand, exhibit a broad range of double-stranded cleavage mechanisms, namely heterodimerization as by BtsI and BbvCI, and sequential cleavage of the dsDNA as monomer as by FokI. These properties have been exploited to create strand-specific nicking enzymes (NEases) (for more information about nicking enzymes, see review in (6)).

## APPLICATIONS UTILIZING RESTRICTION ENZYMES

Traditional Cloning

In combination with DNA ligases, REases facilitated a robust "cut and paste" workflow where a defined DNA fragment could be moved from one organism to another (Fig. 2). Using this methodology, Stanley Cohen and his colleagues incorporated exogenous DNA into natural plasmids to create the vehicle for cloning-plasmid vectors that self-propagate in *E. coli* (7). These became the backbone of many present-day vectors, and enabled the cloning of DNA for the study and production of recombinant proteins. Restriction enzymes are also useful as post-cloning confirmatory tools, to ensure that insertions have taken place correctly. The traditional cloning workflow, along with DNA amplification technologies, such as PCR and RT-PCR, has become a mainstream application for REases and facilitated the study of many molecular mechanisms.

DNA Mapping

Armed with only a handful of REases in the early 1970s, Daniel Nathans mapped the functional units of SV40 DNA (8), and commenced the era of "restriction mapping" and comparison of complex genomes. It has since evolved into sophisticated methodologies that allow the

Figure 2. Traditional Cloning Workflow
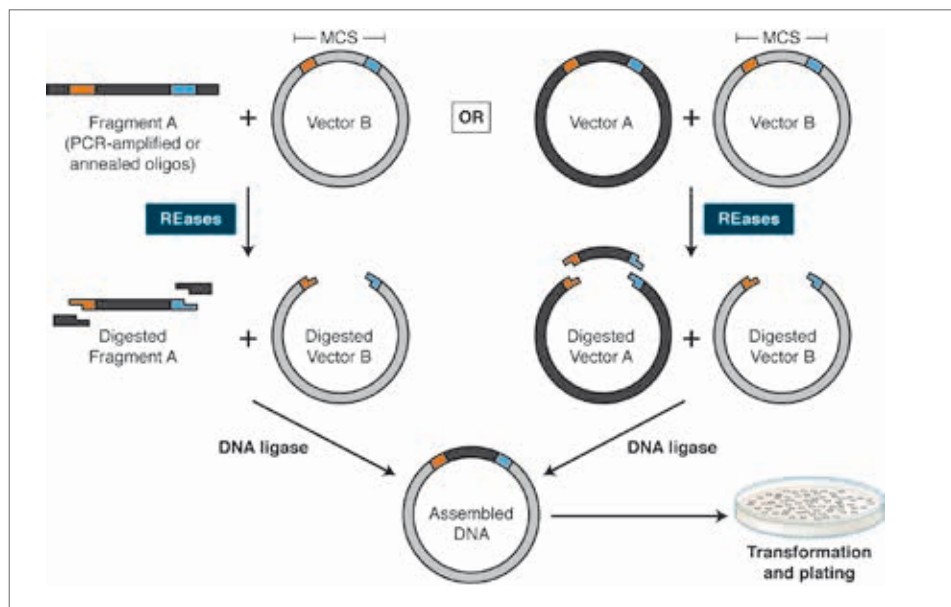
Using PCR, restriction sites are added to both ends of a dsDNA, which is then digested by the corresponding REases. The cleaved DNA can then be ligated to a plasmid vector cleaved by the same or compatible REases with T4 DNA ligase. DNA fragments can also be moved from one vector into another by digesting with REases and ligating to compatible ends of the target vector.

detection of single nucleotide polymorphisms (SNP) and insertions/deletions (Indels) (9), driving applications that include identifying genetic disorder loci, assessing the genetic diversity of populations and parental testing.

Understanding Epigenetic Modifications

REases' sensitivity to the methylation status of target bases has been exploited to map modified bases within genomes. Restriction Landmark Genome Scanning (RLGS) is a 2-dimensional gel electrophoresis-based mapping technique that employs NotI (GC^GGCCGC), AscI (GG^CGCGCC), EagI (C^GGCCG) or BssHII (G^CGCGC) to interrogate changes in the methylation patterns of the genome during the development of normal and cancer cells. Methylation-Sensitive Amplification Polymorphism (MSAP) takes advantage of

the differential sensitivity of MspI and HpaII toward the methylation status of the second C of quadruplet CCGG to identify 5-methylcytosine (5-mC) or 5-hydroxymethylcytosine (5-hmC) (10,11). Scientists at NEB further exploited the property of MspI and HpaII on 5-glucosyl hydroxymethylcytosine (5-ghmC) in the EpiMark® 5-hmC and 5-mC Analysis Kit (NEB #E3317S)(12), which differentiates 5-hmC from 5-mC for more refined epigenetic marker identification and quantitation (for more information, visit EpiMark.com). Additionally, the recently discovered REases that recognize and cleave DNA at 5-mC and 5-hmC sites (e.g., MspJI, FspEI and LpnPI), as well as those that preferentially cleave 5-hmC or 5-ghmC over 5-mC or C (e.g., PvuRtsII, AbaSI) (13), are potential tools for high-throughput mapping of the cytosine-based epigenetic markers in cytosine-methylated genomes (14,15).

DEVELOPMENT OF RESTRICTION ENZYMES AND GENE EDITING TECHNIQUES

1952-53
"Host-induced variation of bacterial viruses" first described

1965-68
Biochemical characterization of the first Type I REases

1970
Purification and characterization of the first Type II REases (HindII/HindIII)

1971-73
Mapping of SV40 DNA

1973-74
Construction of the first autonomously replicating plasmids using EcoRI and DNA ligase (the pSC series)

1975
REBASE (Restriction Enzyme Database) launched

1975
Technique: Restriction Fragment Length Polymorphism (RFLP) analysis

In vitro DNA Assembly Technologies

Synthetic biology is a rapidly growing field, in which defined components are used to create biological systems for the study of biological processes and the creation of useful biological devices (16). Novel technologies such as BioBrick™ originally emerged to facilitate the building of such biological systems. Recently, more robust approaches such as Golden Gate Assembly and Gibson Assembly™ have been widely adopted by the synthetic biology community. Both approaches allow for the parallel and seamless assembly of multiple DNA fragments without resorting to non-standard bases.

BioBrick: The BioBricks community (http://hdl.handle.net/1721.1/21168) sought to create thousands of "standardized parts" of DNAs for rapid gene assembly. With the annual International Genetically Engineered Machines (iGEM) competition (igem.org), the BioBricks community grew and elicited broad interest from many university students in synthetic biology. Based on traditional REase-ligation methodology, BioBrick and its derivative methodologies (BioBrick Assembly Kit, NEB #E0546, and its derivative, BglBricks (17)) are easy to use, but they introduce scar sequences at the junctions. They also require multiple cloning cycles to create a working biological system.
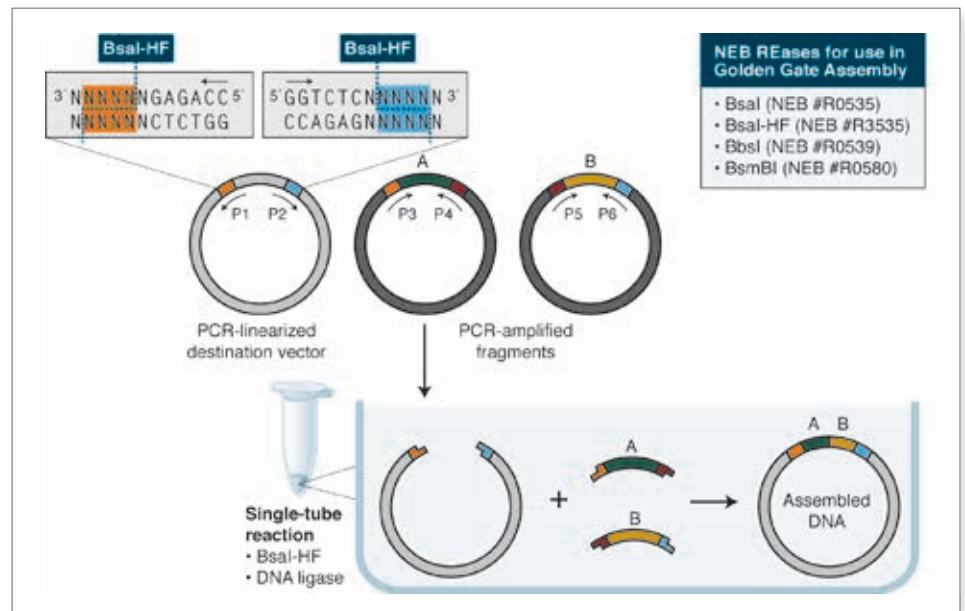
Golden Gate Assembly: Golden Gate Assembly and its derivative methods (19,20) exploit the ability of Type IIS REases to cleave DNA outside of the recognition sequence. The inserts and cloning vectors are designed to place the Type IIS recognition site distal to the cleavage site, such that the Type IIS REase can remove the recognition sequence from the assembly (Fig. 3). The advantages of such an arrangement are three-fold: 1. the overhang sequence created is not dictated by the REase, and therefore no scar sequence is introduced; 2. the fragment-specific sequence of the overhangs allows orderly assembly of multiple fragments simultaneously; 3. and the restriction site is eliminated from the ligated product, so digestion and ligation can be carried out simultaneously. The net result is the ordered and seamless assembly of DNA fragments in one reaction. The accuracy of the assembly is dependent on the length of the overhang sequences. Therefore, Type IIS REases that create 4-base overhangs (such as BsaI/BsaI-HF, BbsI, BsmBI and Esp3I) are preferred. The downside of these Type IIS REase-based methods is that the small number of overhanging bases can lead to the mis-ligation of fragments with similar overhang sequences (21). It is also necessary to verify that the Type IIS REase sites used are not present in the fragments for the assembly of the expected product. Nonetheless, Golden Gate Assembly is a robust technology that generates multiple site-directed mutations (22) and assembles multiple DNA fragments (23,24). As open source methods and reagents have become increasingly available (see www.addgene.org), Golden Gate Assembly has been widely used in the construction of custom-specific TALENs for *in vivo* gene editing (25), among other applications.

Gibson Assembly: Daniel G. Gibson, of the J. Craig Venter Institute, described a robust exonuclease-based method to assembly DNA seamlessly and in the correct order. The reaction is carried out under isothermal conditions using three enzymatic activities: a 5' exonuclease generates long overhangs, a polymerase fills in the gaps of the annealed ss regions, and a DNA ligase seals the nicks of the annealed and filled-in gaps (26) (Fig. 4). Applying this methodology, the 16.3 kb mouse mitochondrial genome was assembled from 600 overlapping 60-mers (26). In combination with *in vivo* assembly in yeast, Gibson Assembly was used to synthesize the

Figure 3. Golden Gate Assembly Workflow

In its simplest form, Golden Gate Assembly requires a BsaI recognition site (GGTCTC) added to both ends of a dsDNA fragment distal to the cleavage site, such that the BsaI site is eliminated by digestion with BsaI or BsaI-HF (GGTCTC 1/5). Upon cleavage, the overhanging sequences of the adjoining fragments anneal to each other. DNA ligase then seals the nicks to create a new covalently linked DNA molecule. Multiple pieces of DNA can be cleaved and ligated simultaneously.


1978
Cloning of the first
R-M system (HhaII)

1983
Cloning of cDNA of Nerve
Growth Factor precursor
from human and mouse

1988
Over 1,000 REases
identified to-date

1978
Over 100 REases
identified to-date

1978
Nobel Prize in Medicine or Physiology awarded to Werner Arber,
Daniel Nathans & Hamilton O. Smith "for the discovery of restriction
enzymes and their application to problems of molecular genetics"
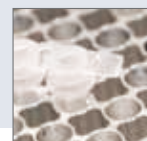
1983-88
Invention and
development of PCR

Figure 4. Gibson Assembly Workflow
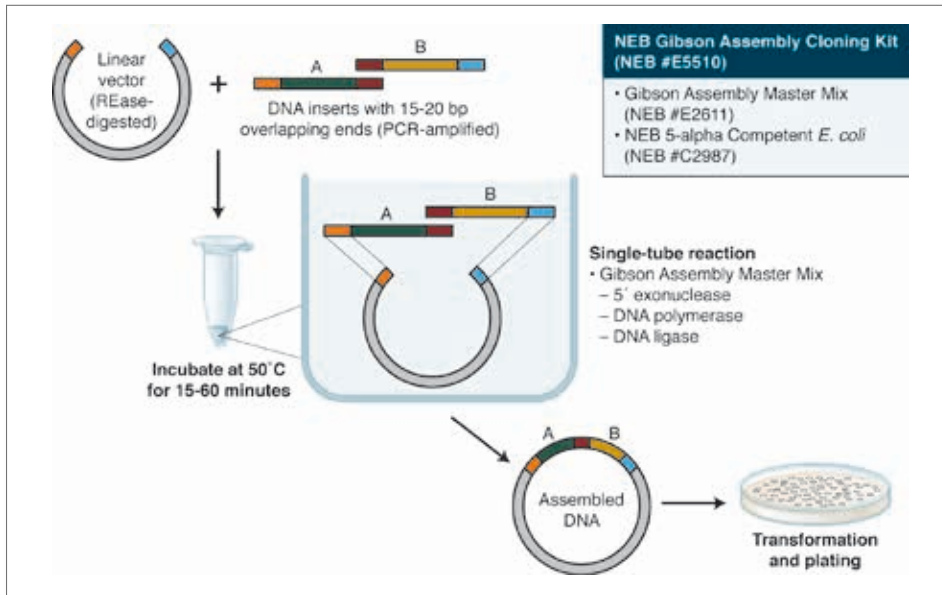
Gibson Assembly employs three enzymatic activities in a single-tube reaction: 5′ exonuclease, the 3′ extension activity of a DNA polymerase and DNA ligase activity. The 5′ exonuclease activity chews back the 5′ end sequences and exposes the complementary sequence for annealing. The polymerase activity then fills in the gaps on the annealed regions. A DNA ligase then seals the nick and covalently links the DNA fragments together. The overlapping sequence of adjoining fragments is much longer than those used in Golden Gate Assembly, and therefore results in a higher percentage of correct assemblies. The NEB Gibson Assembly Master Mix (NEB #E2611) and Gibson Assembly Cloning Kit (NEB #E5510S) enable rapid assembly at 50˚C.

1.1 Mbp *Mycoplasma mycoides* genome. The synthesized genome was transplanted to a *M. capricolum* recipient cell creating new self-replicating *M. mycoides* cells (27).

Gibson Assembly can also be used for cloning; the assembly of a DNA insert with a restriction-digested vector, followed by transformation, can be completed in a little less than two hours with the Gibson Assembly Cloning Kit (NEB #E5510S, for more information, visit NEBGibson.com). Other applications of Gibson Assembly include the introduction of multiple mutations, assembly of plasmid vectors from chemically synthesized oligonucleotides, and creating combinatorial libraries of genes and pathways.

Construction of DNA Libraries

SAGE (Serial Analysis of Gene Expression) has allowed the identification and quantification of a large number of mRNA transcripts. It has been widely used in cancer research to identify mutations and study gene expression. REases are key to the SAGE workflow. NlaIII is instrumental as an anchoring enzyme, because of its unique property of recognizing a 4-bp sequence CATG and creating a 4 nucleotide overhang of the same sequence. The use of Type IIS enzymes as tagging enzymes that cleave further and further away from the recognition sequence allows for the higher information content of SAGE analyses (e.g., FokI and BsmFI in SAGE (28), MmeI in LongSAGE (29) and EcoP15I in SuperSAGE (30) and DeepSAGE (31)).

Chromosome conformation capture (3C) and derivative methods allow the mapping of the spatial organizations of genomes in unprecedentedly high resolution and throughput (32). REases plays an indispensible role in creating the compatible ends of the DNA cross-linked to its interacting proteins, such that spatially associated sequences can be ligated and, hence, identified through high-throughput sequencing.

Although REases do not allow for the random fragmentation of DNA that most next-generation DNA sequencing technologies require, they are being used in novel target enrichment methodologies (hairpin adaptor ligation (33) and HaloPlex™ enrichment (Agilent)). The long-reach REase, AcuI, and USER™ Enzyme are also used to insert tags into sample DNA, which is then amplified by rolling circle amplification (RCA) to form long, single-stranded DNA "nanoballs" that serve as template in the high density, chip-based sequencing-by-ligation methodology, developed by Complete Genomics (34). ApeKI was also used to generate the DNA library for a genotyping-by-sequencing technology for the study of sequence diversity of maize (35).

Creation of Nicks in DNA

Before NEases were available, non-hydrolyzable phosphorothioate groups were incorporated into a specific strand of the target DNA such that REases can introduce sequence- and strand-specific nicks into the DNA for applications such as strand displacement amplification (SDA), where a strand-displacing DNA polymerase (e.g., Bst 2.0 DNA Polymerase, NEB #M0537) extends from the newly created 3'-hydroxyl end, and essentially replicates the complementary sequence (36). Because the nicking site is regenerated, repeated nicking-extension cycles result in amplification of specific single-stranded segments of the sample DNA without the need for thermocycling. NEases greatly streamline the workflow of such applications and open the door to applications that cannot be achieved by REases. Nicking enzyme-based isothermal

DEVELOPMENT OF RESTRICTION ENZYMES AND GENE EDITING TECHNIQUES (CONT'D)

1990-PRESENT
Cloning via PCR amplification followed by REase digestion and ligation

1996
Development of zinc finger nucleases (ZFN)

2000-PRESENT
Gene disruption and insertion induced by ZFNs and engineered meganucleases

1995
Technique: Amplification Fragment Length Polymorphism (AFLP) analysis

1997
Technique: methylation-sensitive amplified polymorphism (MSAP) analysis

2008
Introduction of Golden Gate Assembly and its variants
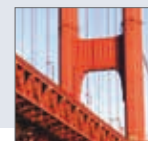
DNA amplification technologies, such as RCA, NESA, EXPAR and related amplification schemes, have been shown to be capable of detecting very low levels of DNA (37,38). Nicking-based DNA amplification had also been incorporated into molecular beacon technologies to amplify signal (39). The implementation of these sample and/or signal amplification schemes can lead to simple, but sensitive and specific, methods for the detection of target DNA molecules in the field (NEAR, EnviroLogix™). By ligating adaptors containing nicking sites to the ends of blunt-ended DNA, the simultaneous actions of the NEase(s) and strand-displacing DNA polymerase can quickly amplify a specific fragment of dsDNA (40). Amplification by nicking-extension cycling is amenable to multiplexing and can potentially achieve a higher fidelity than PCR. The combined activity of NEases and Bst DNA polymerase have also been used to introduce site-specific fluorescent labels into long/chromosomal DNA *in vitro* for visualization (nanocoding) (41). Innovative applications of nicking enzymes include the generation of reporter plasmids with modified bases or structures (42) and the creation of a DNA motor that transports a DNA cargo without added energy (43). A review of NEases and their applications has been published elsewhere (6).

In vivo Gene Editing

The ability to "cut and paste" DNA using REases *in vitro* has naturally led to the quest for performing the art *in vivo* to correct mutations that cause genetic diseases. Direct use of REases and homing endonucleases in Restriction Enzyme Mediated Integration (REMI) facilitated the generation of transgenic embryos of higher organisms (44,45). There is, however, no control over the integration site. The concept of editing genes through site-specific cleavage has been realized using Zinc Finger Nucleases (ZFNs) and Transcription Activator-like Effector Nucleases (TALENs), due to their ability to create customizable double stranded breaks in complex genomes. With the great success of gene editing in model organisms and livestock (46-50), the therapeutic potential of these gene editing reagents is being put to the first test in the Phase I/II clinical trials of a regime that uses a ZFN to improve CD4+ T-cell counts by knocking out the expression of the CCR5 gene in autologous T-cells from HIV patients (ClinicalTrials.gov indentifier NCT00842634) (51). Recent research on CRISPR, the adaptive defense system of bacteria and archaea, has shown the potential of the Cas9-crRNA complex as programmable RNA-guided DNA endonucleases and strand-specific nicking endonucleases for *in vivo* gene editing (52,53).

MOVING FORWARD

Restriction enzymes have been one of the major forces that enabled the cloning of genes and transformed molecular biology. Novel technologies, such as Golden Gate Assembly and Gibson Assembly, continue to emerge and expand our ability to create new DNA molecules. The potential to generate new recognition specificity in the MmeI family REases, the engineering of more NEases and the discovery of ever more modification-specific REases continues to create new tools for DNA manipulation and epigenome analysis. Innovative applications of these enzymes will take REases' role beyond molecular cloning by continuing to accelerate the development of biotechnology and presenting us with new opportunities and challenges.
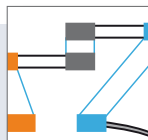
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For more information on restriction endonucleases, visit REBASE (The Restriction Enzyme Database), a comprehensive database of information about all known restriction endonucleases and related proteins.

rebase.neb.com.
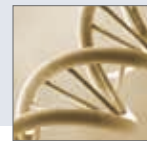
2009 Phase I clinical trial to remove CCR5 from autologous T-cells using ZFNs begins	**2009** Introduction of Gibson Assembly
2009 Identification of the DNA recognition code of TAL effectors	**2010-PRESENT** Gene disruption and insertion induced by TALENs in model organisms
2011 EpiMark 5-hmC and 5-mC Analysis Kit released	**2012** Over 4,000 REases experimentally characterized to-date
	2012 Cas9 enables RNA-guided genome editing