Special Cloning Edition

NEB® EXPRESSIONS

A scientific update from New England Biolabs | Issue III, 2013

Foundations of Molecular Cloning – Past, Present & Future *page 3*

Comparison of Cloning Workflows *page 8*

New Products to Facilitate your Cloning *page 10*

Getting Started with Molecular Cloning *page 14*

NEW ENGLAND BioLabs® Inc.

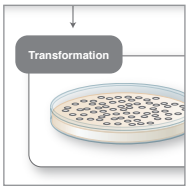
Contents

Issue III, 2013

3 FEATURE ARTICLE

Foundations of Molecular Cloning – Past, Present & Future

Understanding where we've come from is essential to knowing where we're going. Brush up on the history of molecular cloning, and learn where it could take us in the future.



8 FEATURED APPLICATION

Cloning Workflow Comparison

How do you know which cloning workflow is right for you? Our helpful Cloning Workflow Comparison graphic can help you differentiate and decide.

10 NEW PRODUCTS

New Cloning Tools to Enhance your Research

We want you to clone with confidence; that's why we continuously strive to develop best-in-class solutions for your molecular cloning needs.

12 SPECIAL OFFER

Earn rewards with your NEB Cloning Purchase

Q. What's more rewarding than a successful cloning experiment?

A. *Earning rewards for buying your cloning reagents from NEB!*

13 FEATURED PRODUCT

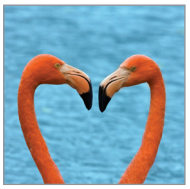
Gibson Assembly® Cloning Kit

Transform your cloning with NEB's Gibson Assembly Cloning Kit. Assemble and clone your insert in just under two hours.

14 TECHNICAL TIPS

Getting Started with Molecular Cloning

Before you dive into your next cloning experiment, read through these useful tips from NEB scientists.



15 APPLICATION NOTE

Robust Colony PCR from Multiple *E. coli* Strains using One*Taq*® Quick-Load® Master Mixes

One*Taq* DNA Polymerase has been formulated for robust yields with minimal optimization. See how it performs in colony PCR with 18 different *E. coli* strains.

COVER PHOTO

Transformed *E. coli* grow on agar plates in a blue/white screening assay.

FEATURE ARTICLE

Foundations of Molecular Cloning – Past, Present & Future

Molecular cloning, a term that has come to mean the creation of recombinant DNA molecules, has spurred progress throughout the life sciences. Beginning in the 1970s, with the discovery of restriction endonucleases – enzymes that selectively and specifically cut molecules of DNA – recombinant DNA technology has seen exponential growth in both application and sophistication, yielding increasingly powerful tools for DNA manipulation. Cloning genes is now so simple and efficient that it has become a standard laboratory technique. This has led to an explosion in the understanding of gene function in recent decades. Emerging technologies promise even greater possibilities, such as enabling researchers to seamlessly stitch together multiple DNA fragments and transform the resulting plasmids into bacteria, in under two hours, or the use of swappable gene cassettes, which can be easily moved between different constructs, to maximize speed and flexibility. In the near future, molecular cloning will likely see the emergence of a new paradigm, with synthetic biology techniques that will enable *in vitro* chemical synthesis of any *in silico*-specified DNA construct. These advances should enable faster construction and iteration of DNA clones, accelerating the development of gene therapy vectors, recombinant protein production processes and new vaccines.

Rebecca Tirabassi, Bitesize Bio.

Introduction

Molecular cloning refers to the isolation of a DNA sequence from any species (often a gene), and its insertion into a vector for propagation, without alteration of the original DNA sequence. Once isolated, molecular clones can be used to generate many copies of the DNA for analysis of the gene sequence, and/or to express the resulting protein for the study or utilization of the protein's function. The clones can also be manipulated and mutated *in vitro* to alter the expression and function of the protein.

The basic cloning workflow includes four steps:

1. Isolation of target DNA fragments (often referred to as inserts)
2. Ligation of inserts into an appropriate cloning vector, creating recombinant molecules (e.g., plasmids)
3. Transformation of recombinant plasmids into bacteria or other suitable host for propagation
4. Screening/selection of hosts containing the intended recombinant plasmid

These four ground-breaking steps were carefully pieced together and performed by multiple laboratories, beginning in the late 1960s and early 1970s. A summary of the discoveries that comprise traditional molecular cloning is described in the following pages.

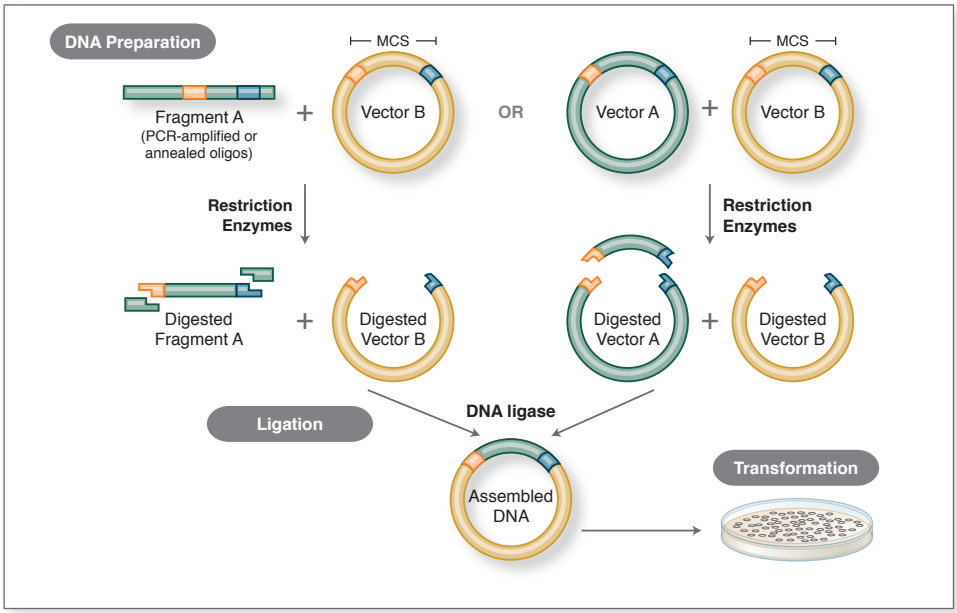
The Foundation of Molecular Cloning

Cutting (Digestion). Recombinant DNA technology first emerged in the late 1960s, with the discovery of enzymes that could specifically cut and join double-stranded DNA molecules. In fact, as early as 1952, two groups independently observed that bacteria encoded a "restriction factor" that prevented bacteriophages from growing within certain hosts (1,2). But the nature of the factor was not discovered until 1968, when Arber and Linn succeeded in isolating an enzyme, termed a restriction factor, that selectively cut exogenous DNA, but not bacterial DNA (3). These studies also identified a methylase enzyme that protected the bacterial DNA from restriction enzymes.

Shortly after Arber and Linn's discovery, Smith extended and confirmed these studies by isolating a restriction enzyme from *Haemophilus influenza*. He demonstrated that the enzyme selectively cut DNA in the middle of a specific 6 base-pair stretch of DNA; one characteristic of certain restriction enzymes is their propensity to cut the DNA substrate in or near specific, often palindromic, "recognition" sequences (4).

The full power of restriction enzymes was not realized until restriction enzymes and gel electrophoresis were used to map the Simian Virus 40 (SV40) genome (5). For these seminal findings, Werner Arber, Hamilton Smith, and Daniel Nathans shared the 1978 Nobel Prize in Medicine.

Figure 1. Traditional Cloning Workflow



Using PCR, restriction sites are added to both ends of a dsDNA, which is then digested by the corresponding restriction enzymes (REases). The cleaved DNA can then be ligated to a plasmid vector possessing compatible ends. DNA fragments can also be moved from one vector into another by digesting with REases and ligating with compatible ends of the target vector. Assembled construct can then be transformed into Escherichia coli (E. coli).

Assembling (Ligation). Much like the discovery of enzymes that cut DNA, the discovery of an enzyme that could join DNA was preceded by earlier, salient observations. In the early 1960s, two groups discovered that genetic recombination could occur though the breakage and ligation of DNA molecules (6,7), closely followed by the observation that linear bacteriophage DNA is rapidly converted to covalently closed circles after infection of the host (8). Just two years later, five groups independently isolated DNA ligases and demonstrated their ability to assemble two pieces of DNA (9-13).

Not long after the discovery of restriction enzymes and DNA ligases, the first recombinant DNA molecule was made. In 1972, Berg separately cut and ligated a piece of lambda bacteriophage DNA or the *E. coli* galactose operon with SV40 DNA to create the first recombinant DNA molecules (14). These studies pioneered the concept that, because of the universal nature of DNA, DNA from any species could be joined together. In 1980, Paul Berg shared the Nobel Prize in Chemistry with Walter Gilbert and Frederick Sanger (the developers of DNA sequencing), for "his fundamental studies of the biochemistry of nucleic acids, with particular regard to recombinant DNA."

Transformation. Recombinant DNA technology would be severely limited, and molecular cloning impossible, without the means to propagate and isolate the newly constructed DNA molecule. The ability to transform bacteria, or induce the uptake, incorporation and expression of foreign genetic material, was first demonstrated by Griffith when he transformed a non-lethal strain of bacteria into a lethal strain by mixing the non-lethal strain with heat-inactivated lethal bacteria (15). However, the nature of the "transforming principle" that conveyed lethality was not understood until 1944. In the same year, Avery, Macleod and McCarty demonstrated that DNA, and not protein, was responsible for inducing the lethal phenotype (16).

Initially, it was believed that the common bacterial laboratory strain, *E. coli*, was refractory to transformation, until Mandel and Higa demonstrated that treatment of *E. coli* with calcium chloride induced the uptake of bacteriophage DNA (17). Cohen applied this principle, in 1972, when he pioneered the transformation of bacteria with plasmids to confer antibiotic resistance on the bacteria (18).

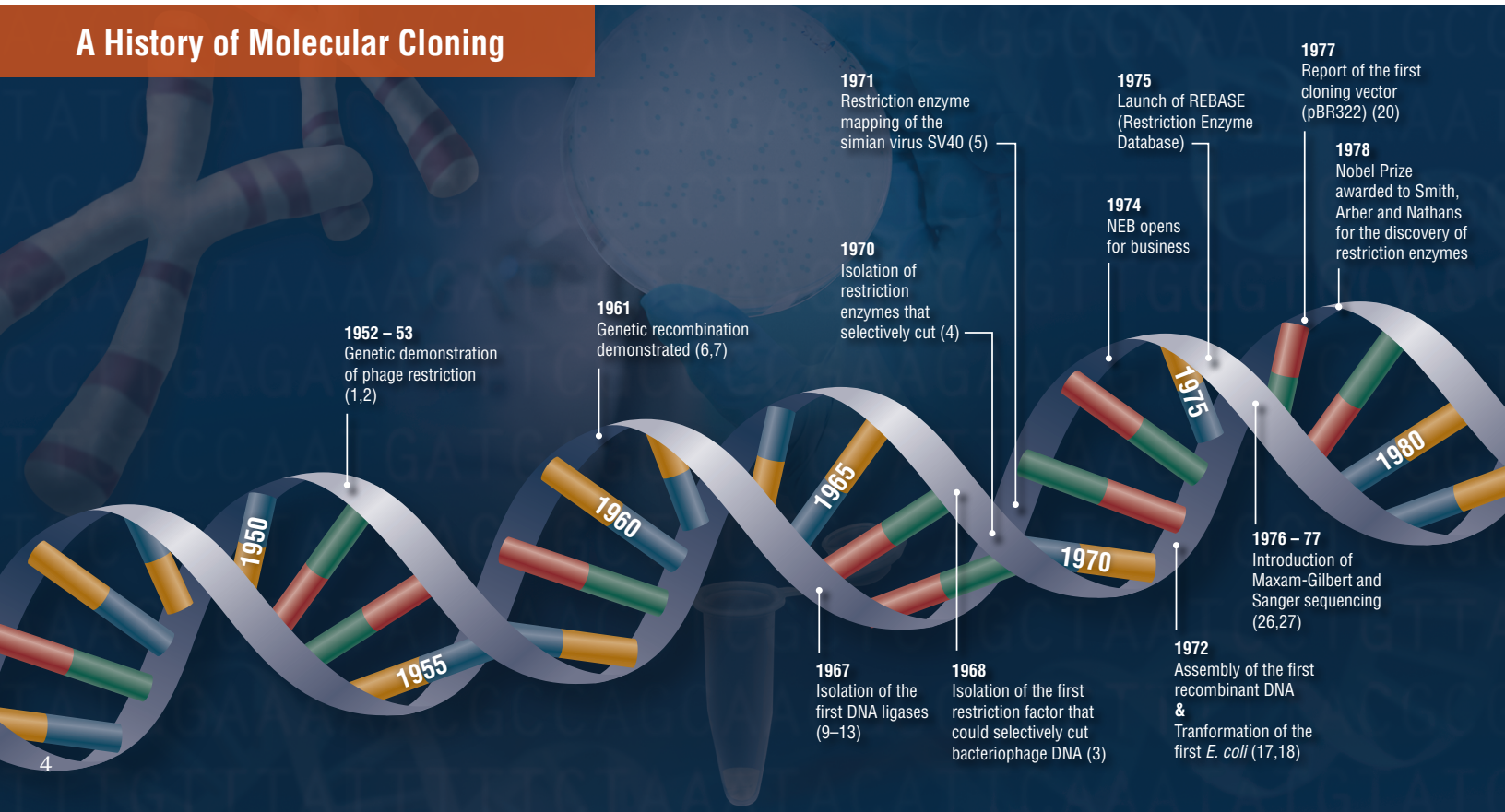
The ultimate experiment: digestion, ligation and transformation of a recombinant DNA molecule was executed by Boyer, Cohen and Chang in 1973, when they digested the plasmid pSC101 with EcoRI, ligated the linearized fragment to another enzyme-restricted plasmid and transformed the resulting recombinant molecule into *E. coli*, conferring tetracycline resistance on the bacteria (19), thus laying the foundation for most recombinant DNA work since.

Building on the Groundwork

While scientists had discovered and applied all of the basic principles for creating and propagating recombinant DNA in bacteria, the process was inefficient. Restriction enzyme preparations were unreliable due to non-standardized purification procedures, plasmids for cloning were cumbersome, difficult to work with and limited in number, and experiments were limited by the amount of insert DNA that could be isolated. Research over the next few decades led to improvements in the techniques and tools available for molecular cloning.

Early vector design.
Development of the first standardized vector. Scientists working in Boyer's lab recognized the need for a general cloning plasmid, a compact plasmid with unique restriction sites for cloning in foreign DNA and the expression of antibiotic resistance genes for selection of transformed bacteria. In 1977, they described the first vector designed for cloning purposes, pBR322 (20). This vector was small, ~4 kilobases in size, and had two antibiotic resistance genes for selection.

A History of Molecular Cloning



Vectors with on-board screening and higher yields. Although antibiotic selection prevented non-transformed bacteria from growing, plasmids that re-ligated without insert DNA fragments (self-ligation) could still confer antibiotic resistance on bacteria. Therefore, finding the correct bacterial clones containing the desired recombinant DNA molecule could be time-consuming.

Vieira and Messing devised a screening tool to identify bacterial colonies containing plasmids with DNA inserts. Based upon the pBR322 plasmid, they created the series of pUC plasmids, which contained a "blue/white screening" system (21). Placement of a multiple cloning site (MCS) containing several unique restriction sites within the LacZ´ gene allowed researchers to screen for bacterial colonies containing plasmids with the foreign DNA insert. When bacteria were plated on the correct media, white colonies contained plasmids with inserts, while blue colonies contained plasmids with no inserts. pUC plasmids had an additional advantage over existing vectors; they contained a mutation that resulted in higher copy numbers, therefore increasing plasmid yields.

Improving restriction digests. Early work with restriction enzymes was hampered by the purity of the enzyme preparation and a lack of understanding of the buffer requirements for each enzyme. In 1975, New England Biolabs (NEB) became the first company to commercialize restriction enzymes produced from a recombinant source. This enabled higher yields, improved purity, lot-to-lot consistency and lower pricing. Currently, over 4,000 restriction enzymes, recognizing over 300 different sequences, have been discovered by scientists across the globe [for a complete list of restriction enzymes and recognition sequences, visit REBASE® at rebase.neb.com (22)]. NEB currently supplies over 230 of these specificities.

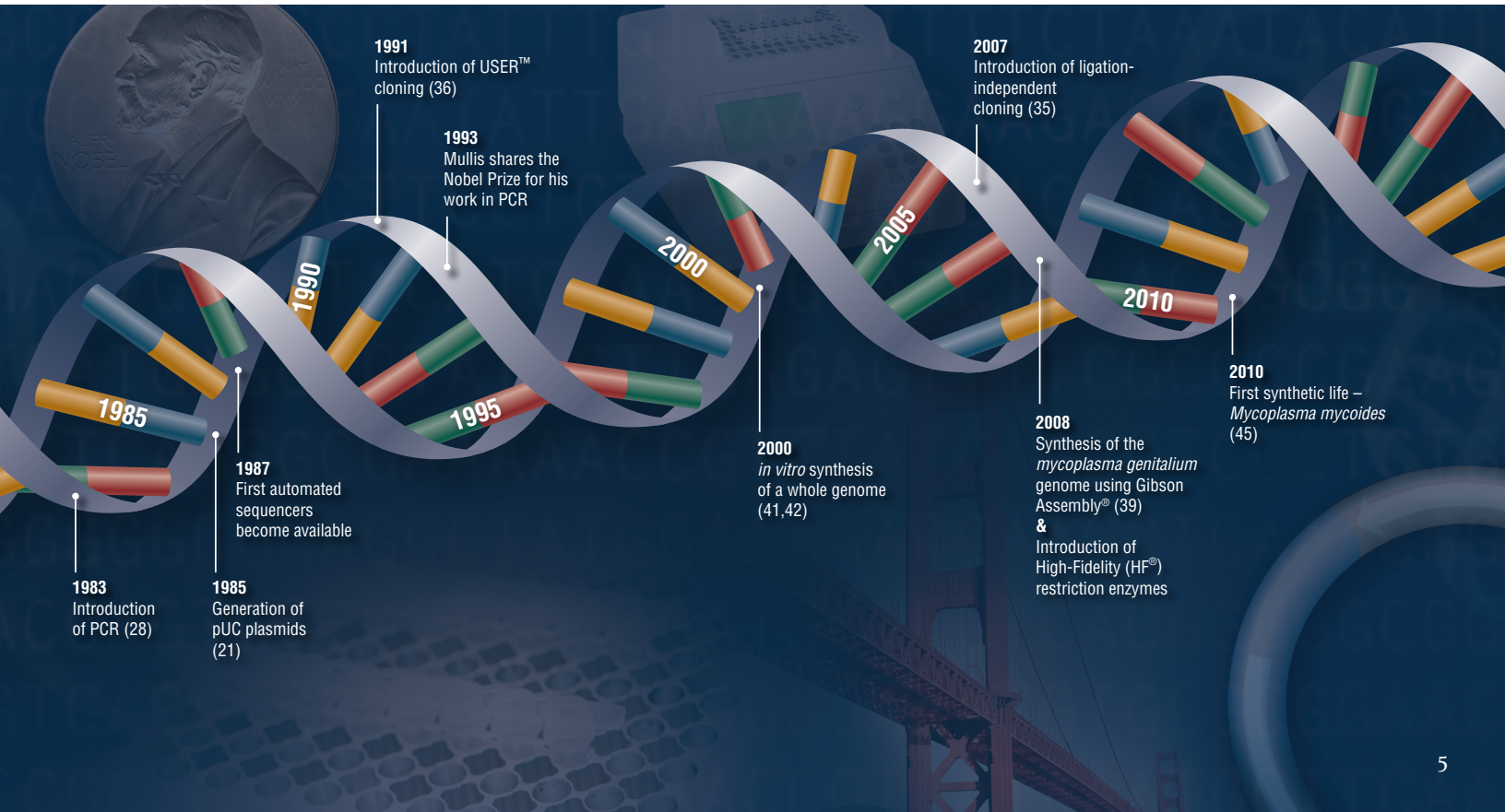
NEB was also one of the first companies to develop a standardized four-buffer system, and to characterize all of its enzyme activities in this buffer system. This led to a better understanding of how to conduct a double digest, or the digestion of DNA with two enzymes simultaneously. Later research led to the development of one-buffer systems, which are compatible with the most common restriction enzymes (such as NEB's CutSmart™ Buffer).

With the advent of commercially available restriction enzyme libraries with known sequence specificities, restriction enzymes became a powerful tool for screening potential recombinant DNA clones. The "diagnostic digest" was, and still is, one of the most common techniques used in molecular cloning.

Vector and insert preparation. Cloning efficiency and versatility were also improved by the development of different techniques for preparing vectors prior to ligation. Alkaline phosphatases were isolated that could remove the 3´ and 5´ phosphate groups from the ends of DNA [and RNA; (23)]. It was soon discovered that treatment of vectors with Calf-Intestinal Phosphatase (CIP) dephosphorylated DNA ends and prevented self-ligation of the vector, increasing recovery of plasmids with insert (24).

The CIP enzyme proved difficult to inactivate, and any residual activity led to dephosphorylation of insert DNA and inhibition of the ligation reaction. The discovery of the heat-labile alkaline phosphatases, such as recombinant Shrimp Alkaline Phosphatase (rSAP) and Antarctic Phosphatase (AP) (both sold by NEB), decreased the steps and time involved, as a simple shift in temperature inactivates the enzyme prior to the ligation step (25).

DNA sequencing arrives. DNA sequencing was developed in the late 1970s when two competing methods were devised. Maxam and Gilbert developed the "chemical sequencing method," which relied on chemical modification of DNA and subsequent cleavage at specific bases (26). At the same time, Sanger and colleagues published on the "chain-termination method", which became



the method used by most researchers (27). The Sanger method quickly became automated, and the first automatic sequencers were sold in 1987.

The ability to determine the sequence of a stretch of DNA enhanced the reliability and versatility of molecular cloning. Once cloned, scientists could sequence clones to definitively identify the correct recombinant molecule, identify new genes or mutations in genes, and easily design oligonucleotides based on the known sequence for additional experiments.

The impact of the polymerase chain reaction. One of the problems in molecular cloning in the early years was obtaining enough insert DNA to clone into the vector. In 1983, Mullis devised a technique that solved this problem and revolutionized molecular cloning (28). He amplified a stretch of target DNA by using opposing primers to amplify both complementary strands of DNA, simultaneously. Through cycles of denaturation, annealing and polymerization, he showed he could exponentially amplify a single copy of DNA. The polymerase chain reaction, or PCR, made it possible to amplify and clone genes from previously inadequate quantities of DNA. For this discovery, Kary Mullis shared the 1993 Nobel Prize in Chemistry "for contributions to the developments of methods within DNA-based chemistry".

In 1970, Temin and Baltimore independently discovered reverse transcriptase in viruses, an enzyme that converts RNA into DNA (29,30). Shortly after PCR was developed, reverse transcription was coupled with PCR (RT-PCR) to allow cloning of messenger RNA (mRNA). Reverse transcription was used to create a DNA copy (cDNA) of mRNA that was subsequently amplified by PCR to create an insert for ligation. For their discovery of the enzyme, Howard Temin and David Baltimore were awarded the 1975 Nobel Prize in Medicine and Physiology, which they shared with Renato Dulbecco.

Cloning of PCR products. The advent of PCR meant that researchers could now clone genes and DNA segments with limited knowledge of amplicon sequence. However, there was little consensus as to the optimal method of PCR product preparation for efficient ligation into cloning vectors.

Several different methods were initially used for cloning PCR products. The simplest, and still the most common, method for cloning PCR products is through the introduction of restriction sites onto the ends of the PCR product (31). This allows for direct, directional cloning of the insert into the vector after restriction digestion. Blunt-ended cloning was developed to directly ligate PCR products generated by polymerases that produced blunt ends, or inserts engineered to have restriction sites that left blunt ends once the insert was digested. This was useful in cloning DNA fragments that did not contain restriction sites compatible with the vector (32).

Shortly after the introduction of PCR, overlap extension PCR was introduced as a method to assemble PCR products into one contiguous DNA sequence (33). In this method, the DNA insert is amplified by PCR using primers that generate a PCR product containing overlapping regions with the vector. The vector and insert are then mixed, denatured and annealed, allowing hybridization of the insert to the vector. A second round of PCR generates recombinant DNA molecules of insert-containing vector. Overlap extension PCR enabled researchers to piece together large genes that could not easily be amplified by traditional PCR methods. Overlap extension PCR was also used to introduce mutations into gene sequences (34).

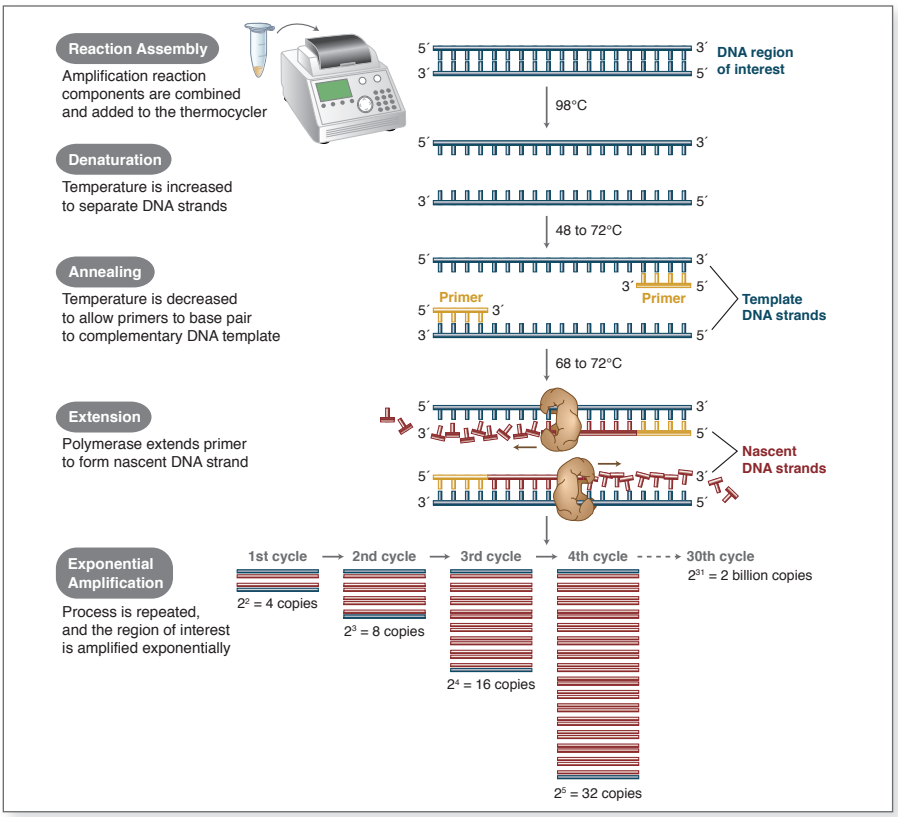
Development of specialized cloning techniques. In an effort to further improve the efficiency of molecular cloning, several specialized tools and techniques were developed that exploited the properties of unique enzymes.

TA Cloning. One approach took advantage of a property of *Taq* DNA Polymerase, the first heat-stable polymerase used for PCR. During amplification, *Taq* adds a single 3´ dA nucleotide to the end of each PCR product. The PCR product can be easily ligated into a vector that has been cut and engineered to contain single T residues on each strand. Several companies have marketed the technique and sell kits containing cloning vectors that are already linearized and "tailed".

LIC. Ligation independent cloning (LIC), as its name implies, allows for the joining of DNA molecules in the absence of DNA ligase. LIC is commonly performed with T4 DNA Polymerase, which is used to generate single-stranded DNA overhangs, >12 nucleotides long, onto both the linearized vector DNA and the insert to be cloned (35). When mixed together, the vector and insert anneal through the long stretch of compatible ends. The length of the compatible ends is sufficient to hold the molecule together in the absence of ligase, even during transformation. Once transformed, the gaps are repaired *in vivo*. There are several different commercially available products for LIC.

USER cloning. USER cloning was first developed in the early 1990s as a restriction enzyme- and ligase-independent cloning method (36). When first conceived, the method relied on using PCR primers that contained a ~12 nucleotide 5´ tail, in which at least four deoxythymidine bases had been substituted with deoxyuridines. The PCR product

Figure 2. Overview of PCR



was treated with uracil DNA glycosidase (UDG) and Endonuclease VIII, which excises the uracil bases and leaves a 3´ overlap that can be annealed to a similarly treated vector. NEB sells the USER enzyme for ligase and restriction enzyme independent cloning reactions.

Future Trends

Molecular cloning has progressed from the cloning of a single DNA fragment to the assembly of multiple DNA components into a single contiguous stretch of DNA. New and emerging technologies seek to transform cloning into a process that is as simple as arranging "blocks" of DNA next to each other.

DNA assembly methods. Many new, elegant technologies allow for the assembly of multiple DNA fragments in a one-tube reaction. The advantages of these technologies are that they are standardized, seamless and mostly sequence independent. In addition, the ability to assemble multiple DNA fragments in one tube turns a series of previously independent restriction/ligation reactions into a streamlined, efficient procedure.

Different techniques and products for gene assembly include SLIC (Sequence and Ligase Independent Cloning), Gibson Assembly (NEB), GeneArt® Seamless Cloning (Life Technologies) and Gateway® Cloning (Invitrogen) (35,37,38).

In DNA assembly, blocks of DNA to be assembled are PCR amplified. Then, the DNA fragments to be assembled adjacent to one another are engineered to contain blocks of complementary sequences that will be ligated together. These could be compatible cohesive ends, such as those used for Gibson Assembly, or regions containing recognition sites for site-specific recombinases (Gateway). The enzyme used for DNA ligation will recognize and assemble each set of compatible regions, creating a single, contiguous DNA molecule in one reaction.

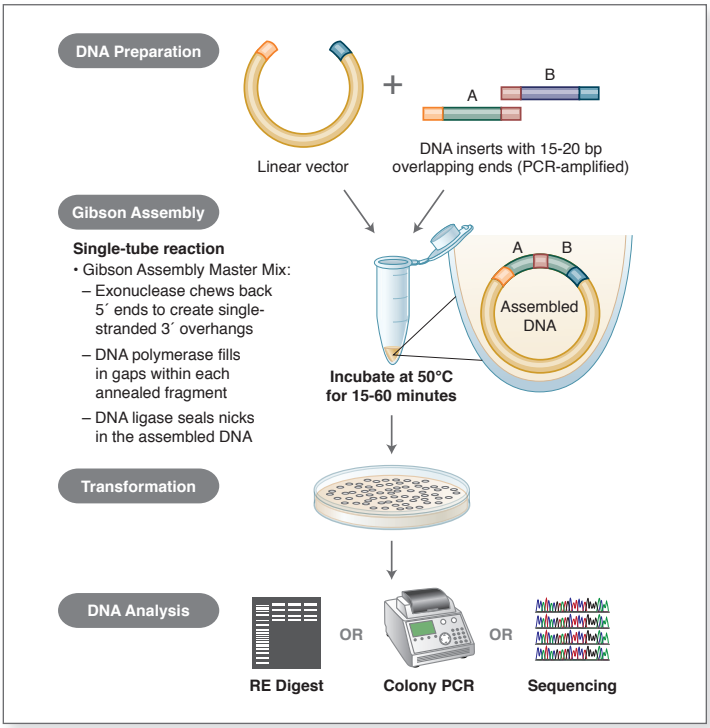
Synthetic biology. DNA synthesis is an area of synthetic biology that is currently revolutionizing recombinant DNA technology. Although a complete gene was first synthesized *in vitro* in 1972 (40), DNA synthesis of large DNA molecules did not become a reality until the early 2000s, when researchers began synthesizing whole genomes *in vitro* (41,42). These early experiments took years to complete, but technology is accelerating the ability to synthesize large DNA molecules.

Conclusion

In the last 40 years, molecular cloning has progressed from arduously isolating and piecing together two pieces of DNA, followed by intensive screening of potential clones, to seamlessly assembling up to 10 DNA fragments with remarkable efficiency in just a few hours, or designing DNA molecules *in silico* and synthesizing them *in vitro*. Together, all of these technologies give molecular biologists an astonishingly powerful toolbox for exploring, manipulating and harnessing DNA, that will further broaden the horizons of science. Among the possibilities are the development of safer recombinant proteins for the treatment of diseases, enhancement of gene therapy (43), and quicker production, validation and release of new vaccines (44). But ultimately, the potential is constrained only by our imaginations.

Rebecca Tirabassi is an Assistant Editor at Bitesizebio.com.

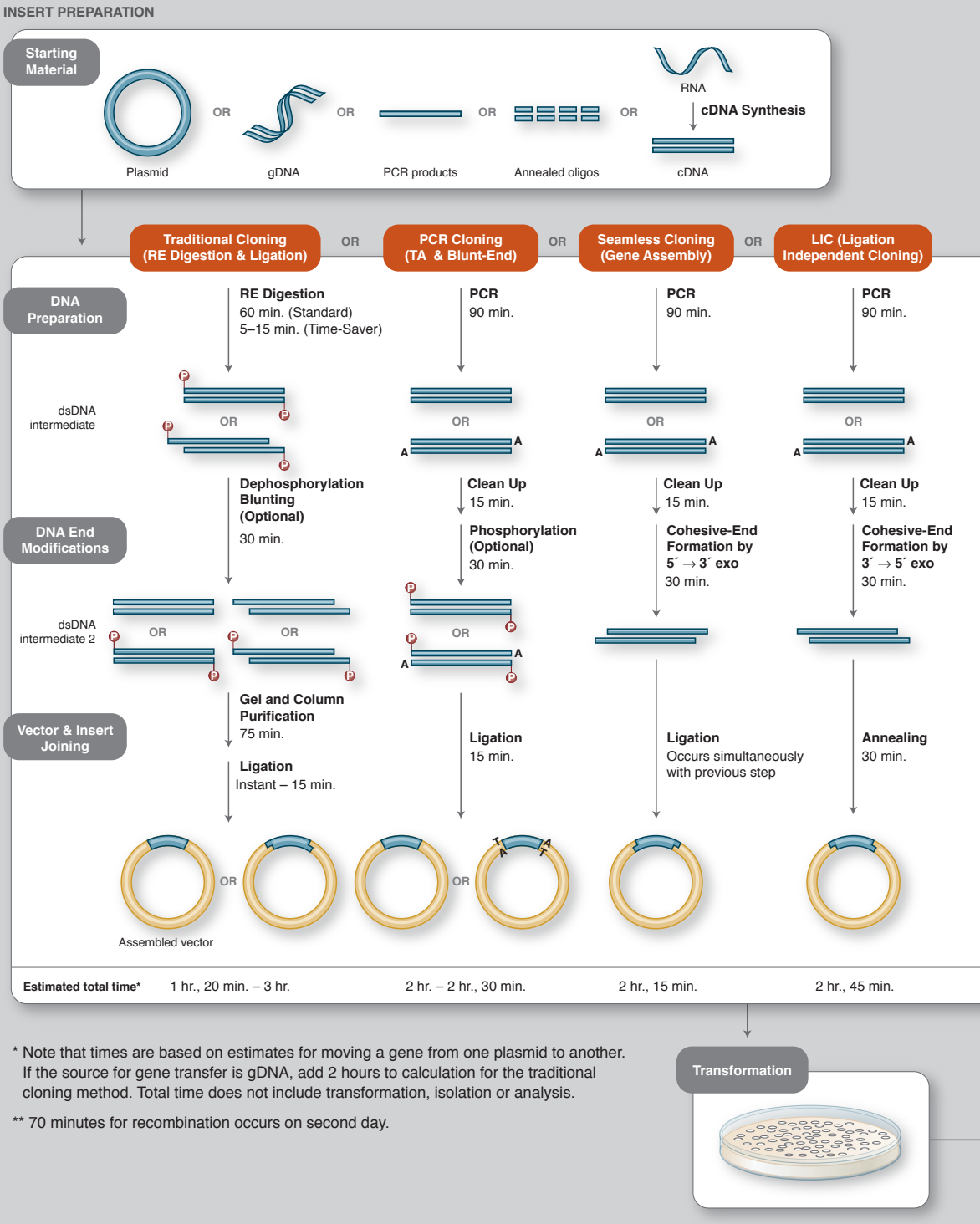
Figure 3. Overview of the Gibson Assembly Cloning Method



References
1. Bertani, G. and Weigle, J.J. (1953) *J. Bacteriol.* 65, 113–121.
2. Luria, S.E. and Human, M.L. (1952) *J. Bacteriol.* 64, 557–569.
3. Linn, S. and Arber, W. (1968) *Proc. Natl. Acad. Sci. USA* 59, 1300–1306.
4. Smith, H.O. and Wilcox, K.W. (1970) *J. Mol. Biol.* 51, 379–391.
5. Danna, K. and Nathans, D. (1971) *Proc. Natl. Acad. Sci. USA* 68, 2913–2917.
6. Kellenberger, G., Zichichi, M.L. and Weigle, J.J. (1961) *Proc. Natl. Acad. Sci. USA* 47, 869–878.
7. Meselson, M. and Weigle, J.J. (1961) *Proc. Natl. Acad. Sci. USA* 47, 857–868.
8. Bode, V.C. and Kaiser, A.D. (1965) *J. Mol. Biol.* 14, 399–417.
9. Cozzarelli, N.R., Melechen, N.E., Jovin, T.M. and Kornberg, A. (1967) *Biochem. Biophys. Res. Commun.* 28, 578–586.
10. Gefter, M.L., Becker, A. and Hurwitz, J. (1967) *Proc. Natl. Acad. Sci. USA* 58, 240–247.
11. Gellert, M. (1967) *Proc. Natl. Acad. Sci. USA* 57, 148–155.
12. Olivera, B.M. and Lehman, I.R. (1967) *Proc. Natl. Acad. Sci. USA* 57, 1426–1433.
13. Weiss, B. and Richardson, C.C. (1967) *Proc. Natl. Acad. Sci. USA* 57, 1021–1028.
14. Jackson, D.A., Symons, R.H. and Berg, P. (1972) *Proc. Natl. Acad. Sci. USA* 1972, 69, 2904–2909.
15. Griffith, F. (1928) *J. Hyg.* 27, 113–159.
16. Avery, O.T., Macleod, C.M. and McCarty, M. (1944) *J. Exp. Med.* 1944, 79, 137–158.
17. Mandel, M. and Higa, A. (1970) *J. Mol. Biol.* 1970, 53:159–162.
18. Cohen, S.N., Chang, A.C. and Hsu, L. (1972) *Proc. Natl. Acad. Sci. USA* 69, 2110–2114.
19. Cohen, S.N., Chang, A.C., Boyer, H.W. and Helling, R.B. (1973) *Proc. Natl. Acad. Sci. USA* 70, 3240–3244.
20. Bolivar, F. et al. (1977) *Gene* 2, 95–113.
21. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene* 33, 103–119.
22. Roberts, R.J., Vincze, T., Posfai, J. and Macelis, D. (2010) *Nucleic Acids Res.* 38, D234–D236.
23. Mossner, E., Boll, M. and Pfleiderer, G. (1980) *Hoppe Seylers Z. Physiol. Chem.* 361, 543–549.
24. Green, M. and Sambrook, J. (2012). *Molecular Cloning: A Laboratory Manual,* (4th ed.), (pp. 189–191). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
25. Rina, M. et al. (2000) *Eur. J. Biochem.* 267, 1230–1238.
26. Maxam, A.M. and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* 74, 560–564.
27. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
28. Mullis, K.B. and Faloona, F.A. (1987) *Methods Enzymol.* 155, 335–350.
29. Baltimore, D. (1970) *Nature* 226, 1209–1211.
30. Temin, H.M. and Mizutani, S. (1970) *Nature* 226:1211–1213.
31. Kaufman, D.L., and Evans, G.A. (1990) *Biotechniques* 9, 304, 306
32. Scharf, S.J., Horn, G.T. and Erlich, H.A. (1986) *Science* 233, 1076–1078.
33. Horton, R.M. et al. (1989) *Gene* 77, 61–68.
34. Ho, S.N. et al. (1989) *Gene* 77, 51–59.
35. Li, M.Z. and Elledge, S.J. (2007) *Nat. Methods* 4, 251–256.
36. Nisson, P.E., Rashtchian, A. and Watkins, P.C. (1991) *PCR Methods Appl.* 1, 120–123.
37. Gibson, D.G. et al. (2009) *Nat. Methods* 6, 343–345.
38. Hartley, J.L., Temple, G.F. and Brasch, M.A. (2000) *Genome Res.* 10, 1788–1795.
39. Gibson, D.G. et al. (2008) *Science* 319, 1215–1220.
40. Agarwal, K.L. et al. (1970) *Nature* 227, 27–34.
41. Blight, K.J., Kolykhalov, A.A. and Rice, C.M. (2000) *Science* 290 1972–1974.
42. Couzin, J. (2002) *Science* 297, 174–175.
43. Hackett, P.B., Largaespada, D.A. and Cooper, L.J. (2010) *Mol. Ther.* 18, 674–683.
44. Dormitzer, P.R. et al. (2013) *Sci. Transl. Med.* 5, 185ra68.
45. Gibson, D.G. et al. (2010) *Science* 329, 52–56.

Cloning Workflow Comparison

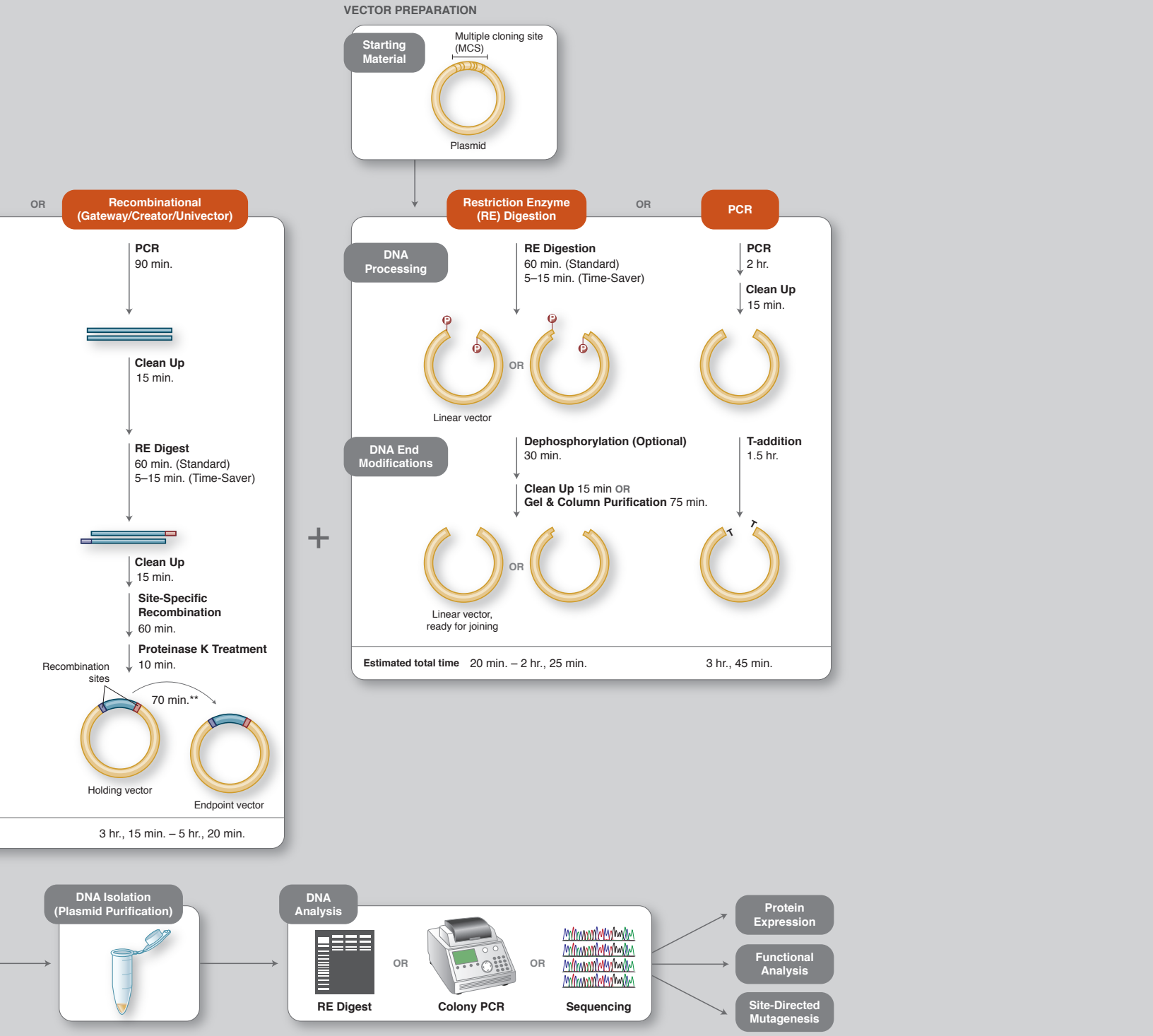
From traditional to advanced molecular cloning techniques, NEB has the right solution for you. Our high-quality reagents are available for every step in the workflow, whether it be specialized enzymes, competent cells or novel solutions – such as Gibson Assembly. Educational tools and technical support are available to you each step of the way, ensuring that you can clone with confidence. When you think of cloning, think NEB!



* Note that times are based on estimates for moving a gene from one plasmid to another. If the source for gene transfer is gDNA, add 2 hours to calculation for the traditional cloning method. Total time does not include transformation, isolation or analysis.

** 70 minutes for recombination occurs on second day.

The figure below compares the various cloning methodologies. To learn more about each of these workflows, as well as the portfolio of products available from NEB, visit **CloneWithNEB.com**.



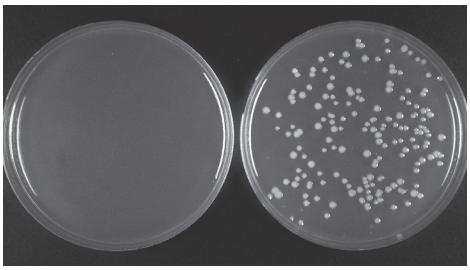
New Cloning Tools to Enhance Your Research

COMING SOON

NEB PCR Cloning Kit

The NEB PCR Cloning Kit allows quick and simple cloning of all your PCR amplicons, regardless of the polymerase used. This kit utilizes a novel mechanism for background colony suppression, and allows for direct cloning from your reaction with no purification step. Enjoy faster cloning with more flexible conditions.

PCR cloning with no background



A 500 bp PCR product incubated with the linearized vector in a 3:1 ratio according to recommended protocol. 2 µl of reaction was transformed into provided NEB 10-beta Competent E. coli and 1/20th of the outgrowth was plated. The left plate serves as the control, with vector backbone only. The right plate contains PCR insert.

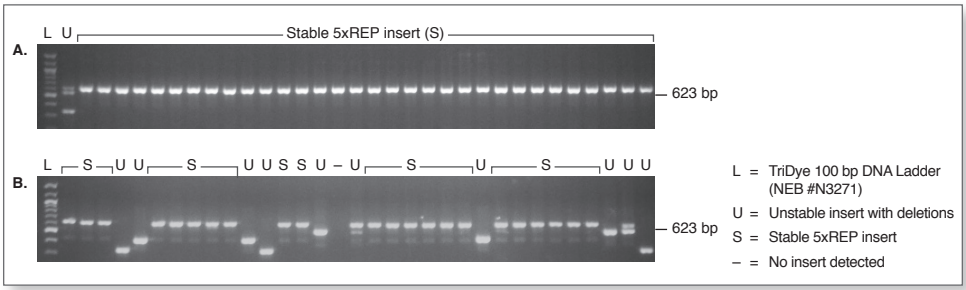
ADVANTAGES

- Works with both blunt and TA ends
- Clone faster, with low/no background
- Get the colonies you need, with high transformation efficiency
- No need for end-modification steps
- No purification step required

NEB Stable Competent *E. coli*

NEB Stable Competent *E. coli* is the ideal strain for cloning unstable inserts, such as direct or inverted repeats. It is the recommended host strain for cloning genes into retroviral/lentiviral vectors. NEB Stable Competent *E. coli* offers high efficiencies and is competitively priced.

NEB Stable enables the isolation of plasmid clones containing repetitive DNA elements



Plasmid pUC-5xREP contains five 32 bp repeats, making it unstable in a recombination-proficient strain. (A) NEB Stable competent cells or (B) Stbl3 competent cells (Invitrogen) were transformed with 2 µl of a pUC-5xREP Gibson Assembly reaction containing 2.2 ng (0.00125 pmol) pUC19 vector and approximately 1 ng (0.0028 pmol) 5xREP insert. Transformed cultures were plated on LB plates containing 100 µg/ml ampicillin and incubated overnight at 30°C. The next day, colony PCR was performed using M13/pUC polylinker primers to analyze 5xREP insert stability. This figure shows the results of analyzing 33 independent colonies.

ADVANTAGES

- Improved cloning of direct repeats and inverted repeats
- Recommended host strain for cloning genes into retroviral/lentiviral vectors
- Free of animal products
- Value pricing

COMING SOON

NEB Essential Cloning Set

The NEB Essential Cloning Set contains a selection of our most popular cloning products, designed to make your cloning experiments even easier. Supplied with a customized NEB cloning box for storage, it's a complete solution for your cloning needs. Don't waste time ordering separately; save money with this convenient kit.

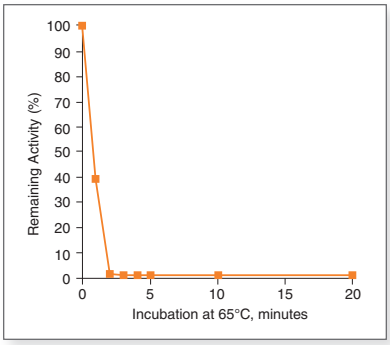
Kit contains:

- Q5® High-Fidelity 2X Master Mix
- Quick-Load Purple 2-Log Ladder
- NEB 5-alpha Competent *E. coli*
- Quick Ligation™ Kit
- rSAP
- Quick Blunting™ Kit
- Coupon for 2 restriction enzymes
- Cloning Storage Box

rSAP

DNA phosphorylation is a key step in the cloning workflow. For your next cloning experiment, consider Shrimp Alkaline Phosphatase (rSAP). rSAP is a heat labile alkaline phosphatase purified from a recombinant source. rSAP contains no affinity tags or other modifications.

rSAP heat inactivation at 65°C



1 unit of rSAP was incubated under recommended reaction conditions, including DNA, for 30 minutes, and then heated at 65°C. Remaining phosphatase activity was measured by PNPP assay.

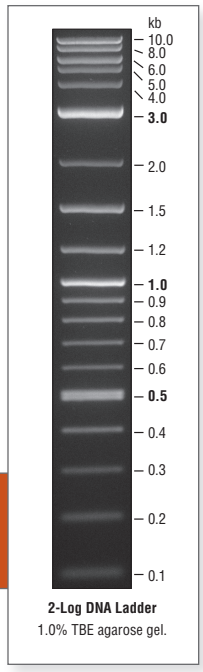
ADVANTAGES

- Inactivate rSAP completely, in 5 minutes at 65°C
- Experience significantly improved stability, compared to native enzyme
- No need for supplemental additives, such as zinc
- Add directly to restriction enzyme digests; active in all NEBuffers
- Use less enzyme; very high specific activity compared to mammalian alkaline phosphatases

Quick-Load Purple 2-Log DNA Ladder Gel Loading Dye, Purple (6X)

NEB's popular 2-Log DNA Ladder is now available in a Quick-Load format for direct gel loading. This product utilizes our new Gel Loading Dye, Purple, which results in brighter, sharper bands when compared to glycerol-based dyes. Additionally, this new dye leaves no UV shadow. Try it, and see how it stacks up.

NOW AVAILABLE
Quick-Load Purple 2-Log DNA Ladder
Now with purple dye



2-Log DNA Ladder
1.0% TBE agarose gel.

ADVANTAGES

- Analyze DNA fragments from 0.1–10 kb
- Convenient, ready-to-load format
- Tighter, brighter bands with new dye format
- COMING SOON – free tube of Gel Loading Dye, Purple with all HF restriction enzymes

Ordering Information

PRODUCT	NEB #	SIZE
NEB Essential Cloning Set	E1400S	1 set
Quick-Load Purple 2-Log DNA Ladder	N0550S	125–250 gel lanes
Gel Loading Dye, Purple (6X)	B7024S	4 ml
NEB PCR Cloning Kit	E1202S	20 rxns
Shrimp Alkaline Phosphatase (rSAP)	M0371S/L	500/2500 units
NEB Stable Competent *E. coli* (High Efficiency)	C3040H	20 x 0.05 ml/tube

SPECIAL OFFER

Earn Rewards with your NEB Cloning Purchases

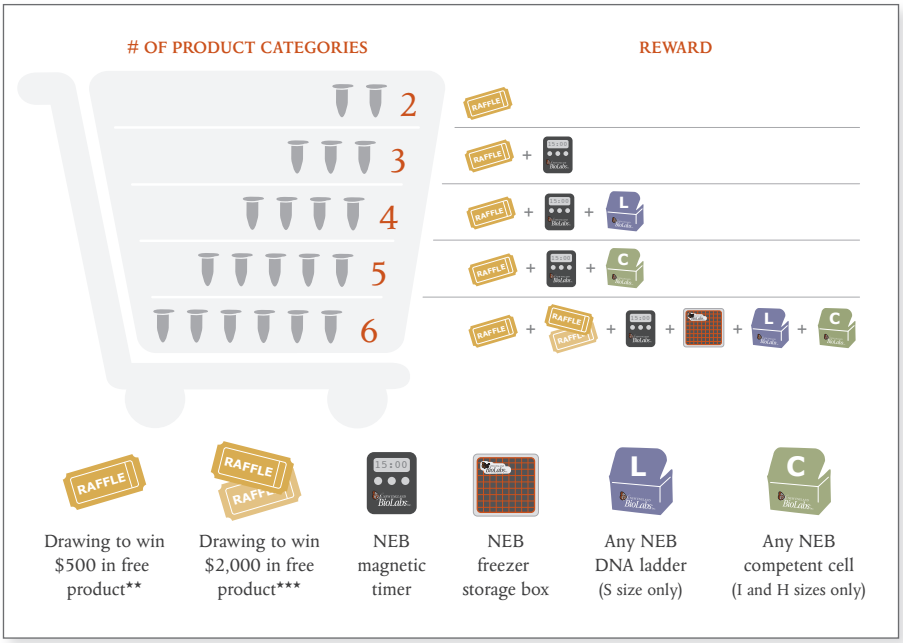
Are you already purchasing restriction enzymes, but haven't tried our ligases? Did you know that NEB supplies competent cells with high transformation efficiencies at value pricing? Now is your chance to try some of NEB's other product areas for cloning and get rewarded for your purchase.

Through February 28, 2014, purchase NEB products from multiple product categories for cloning and be eligible for rewards.* (Offer valid U.S. only)

Choose from the following product areas:

- Cloning competent cells
- DNA ladders
- DNA ligases
- PCR polymerases
- DNA phosphatases
- Restriction enzymes

Visit **www.CloneWithConfidence.com**, and enter the NEB order confirmation number for the order containing the relevant product category. You may use the same order number more than once if the order contains products from more than one category. You will receive an email with instructions on how to redeem your reward, or continue collecting to earn rewards from higher tiers. Contact us at **clone@neb.com** for any questions regarding this offer.



* For promotion terms & conditions, visit CloneWithConfidence.com

** One drawing to be held every month during promotion period. Winner will be notified by email.

*** One drawing to be held on March 1, 2014. Winner will be notified by email.

TOOLS & RESOURCES

Visit ClonewithNEB.com to find:

- The full list of products available for molecular cloning
- Protocols & FAQs
- Online tutorials

Learn about recommended products for cloning, by downloading our Reagents and Tools for Molecular Cloning Brochure.

Download the latest Cloning Technical Guide for help with product selection, protocols, tips for optimization and troubleshooting.

FEATURED PRODUCT

Gibson Assembly Cloning Kit

New England Biolabs has revolutionized your laboratory's standard cloning methodology. The Gibson Assembly Cloning Kit combines the power of the Gibson Assembly Master Mix with NEB 5-alpha Competent *E. coli,* enabling fragment assembly and cloning in just under two hours. Save time, without sacrificing efficiency.

Gibson Assembly was developed by Dr. Daniel Gibson and his colleagues at the J. Craig Venter Institute, and licensed to NEB by Synthetic Genomics, Inc., and allows for successful assembly of multiple DNA fragments, regardless of fragment length or end compatibility.

Gibson Assembly efficiently joins multiple overlapping DNA fragments in a single-tube isothermal reaction. The Gibson Assembly Master Mix includes three different enzymatic activities that perform in a single buffer:

- The exonuclease creates a single-stranded 3´ overhang that facilitates the annealing of fragments that share complementarity at one end
- The polymerase fills in gaps within each annealed fragment
- The DNA ligase seals nicks in the assembled DNA

Resulting DNA is ready to be transformed. The full workflow is illustrated on page 7.

Making ends meet is now quicker and easier than ever before, with the Gibson Assembly Cloning Kit from NEB.

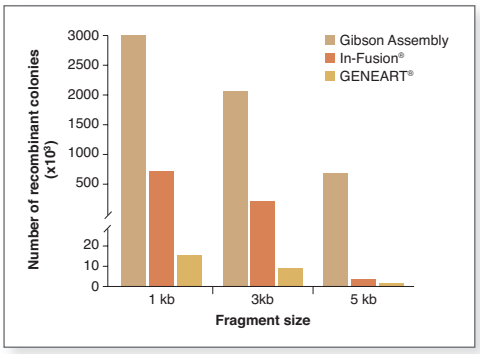
ADVANTAGES

- Rapid cloning into any vector with no additional sequence added
- Easy-to-use protocols enable cloning and transformation in just under two hours
- High efficiencies, even with assembled fragments up to 20 kb
- Includes competent cells
- Use NEBuilder®, our online primer design tool, to design primers for your Gibson Assembly reaction

Some components of this product are manufactured by New England Biolabs, Inc. under license from Synthetic Genomics, Inc.

SGHDNA

Gibson Assembly Cloning Kit provides robust transformation efficiencies



Assembly reactions containing 25 ng of linear pUC19 vector and 0.04 pmol of each fragment were performed following individual suppliers' recommended protocols and using their competent cells. The total number of recombinant colonies was calculated per 25 ng of linear pUC19 vector added to the assembly reaction.

Ordering Information

PRODUCT	NEB #	SIZE
Gibson Assembly Cloning Kit	E5510S	10 rxns

TECHNICAL TIPS

FAQ Spotlight

Q: What is the difference between the Gibson Assembly Master Mix (NEB #E2611) and the Gibson Assembly Cloning Kit (NEB #E5510)?

A: *Both products are supplied with the same Gibson Assembly Master Mix. The Gibson Assembly Cloning Kit also includes NEB 5-alpha Competent E. coli.*

Q: What incubation times are recommended for an assembly reaction?

A: *For assembling 2–3 fragments, 15 minute incubation times are sufficient. For assembling 4–6 fragments, 60 minute incubation times are recommended. Reaction times less than 15 minute are generally not recommended.*

Getting Started with Molecular Cloning: Simple Guidelines to Improve your Cloning Efficiency

Molecular cloning has traditionally utilized restriction enzymes to excise a fragment of interest from source DNA, and to linearize a plasmid vector while creating compatible ends. After purification of the insert and vector, both are joined with the activity of a DNA ligase, and the newly-created recombinant vector is used to transform an *E. coli* host for propagation of the recombinant molecule. More recently, PCR has been used to generate both the vector and insert, which can be joined using a variety of techniques, ranging from standard DNA ligation or enzymatic joining using a recombinase or topoisomerase, to homologous recombination. These newly-fashioned recombinant constructions may then be used to transform an appropriate *E. coli* host.

Regardless of which cloning method is chosen, the process can be made more efficient by following good practices in the lab. The following tips will help improve the success of your cloning experiments.

1 Take the time to plan your experiments

Attention to detail when planning a cloning project is essential. Ensure that your design is sound with a complete understanding of the methods being used and the sequences being generated. Pay attention to the junction sequences and the effect on reading frames of any translated sequences. Check both the vector and insert for internal restriction sites (we recommend NEBcutter®; http://tools.neb.com/NEBcutter2/index.php) prior to designing PCR primers that contain similar sites to those used for cloning. Verify that the antibiotic selective marker in the vector is compatible with the chosen host strain.

2 Start with clean DNA at the right concentration

Ensuring that your source DNA is free of contaminants, including nucleases and unwanted enzymatic activities, is important. Using commercially-available spin columns to purify starting DNA is good practice. Completely remove solvents, such as phenol, chloroform and ethanol, prior to manipulation of the DNA. Ensure that the final elution of DNA from the spin columns is made with salt-free buffer to prevent inhibition of the downstream steps, either restriction digestion or PCR amplification. Use a sufficient amount of DNA for the technique being used. Preparative restriction digests often require between 0.2 –2.0 µg, while only single nanogram amounts are usually sufficient for DNA being used as a template for PCR.

3 Perform your restriction digests carefully

It is important to set up digestion reactions properly. The volume of the reaction should be compatible with the downstream step, for instance, smaller than the volume of the well of an agarose gel used to resolve the fragments. For a typical cloning reaction, this is often between 20–50 µl. The volume of restriction enzyme(s) added should be no more than 10% of the total reaction volume, to ensure that the glycerol concentration stays below 5%; this is an important consideration to minimize star activity, or unwanted cleavage.

4 Mind your ends

DNA ends prepared for cloning by restriction digest are ready for ligation without further modification, assuming the ends to be joined are compatible (have complementary overhangs or are blunt). If the ends are non-compatible, modify them using the appropriate end-modification method (e.g., use of blunting reagents, phosphatases, etc.).

DNA ends prepared by PCR for cloning may have a 3′ addition of a single adenine (A) residue as a result of amplification using *Taq* DNA Polymerase (NEB #M0273). High-fidelity DNA polymerases, such as Q5 (NEB #M0491), leave blunt ends. PCR using standard commercial primers produces non-phosphorylated fragments, unless the primers were 5′ phosphorylated. The PCR product may need to be kinase treated to add a 5′ phosphate prior to ligation with a dephosphorylated vector.

5 Clean up your DNA prior to vector:insert joining

For low-throughput projects, such as single gene cloning, you'll want to clean up your digest, end treatment or PCR reaction prior to proceeding. This can be achieved with gel electrophoresis or spin columns. Isolating the desired DNA species and resolving it from unwanted parent vectors and/or other DNA fragments can dramatically improve your cloning results.

Confirm digested DNA on an agarose gel prior to ligation. For a single product, run a small amount of the digest, and then use a spin column to capture the remainder. When there are multiple fragments produced and only one is to be used, resolving the fragments on a gel and excising the desired fragment under UV light is common. Using longwave (365nm) UV light will minimize any radiation-induced DNA damage to the fragment of interest. The DNA fragment may then be recovered from the agarose slice with a gel extraction kit or β-Agarase I (NEB #M0392).

6 Quantitate your isolated material

Simple quantitation methods, such as gel electrophoresis with mass standards or spectroscopic quantitation on low-input spectrophotometers (such as a NanoSpec®), ensure that the proper amount of material is used for the downstream joining reaction.

7 Follow the manufacturer's guidelines for the joining reaction

For traditional cloning, follow the guidelines specified by the ligase supplier. If a 3:1 molar ratio of insert to vector is recommended, try this first for the best result. Using a 3:1 mass ratio is not the same thing (unless the insert and vector have the same mass). Ligation usually proceeds quickly and, unless your cloning project requires the generation of a high-complexity library that benefits from the absolute capture of every possible ligation product, long incubation times are not necessary.

Follow the manufacturers' guidelines for the joining reactions in PCR cloning and seamless cloning. If you are performing a cloning protocol for the first time, adhere to the recommended protocol for optimal results.

8 Use competent cells that are suited to your needs

While some labs have traditionally prepared their own competent cells "from scratch", the levels of competence achieved rarely matches the high levels attained with commercially-available competent cells. Commercially-available competent cells save time and resources, and make cloning more reproducible.

TECHNICAL TIPS

Robust Colony PCR from Multiple *E. coli* Strains using One*Taq* Quick-Load Master Mixes

Yan Xu, Ph.D., New England Biolabs, Inc.

Introduction

Colony PCR is a commonly used method to quickly screen for plasmids containing a desired insert directly from bacterial colonies. This method eliminates the need to culture individual colonies and prepare plasmid DNA before analysis. However, the presence of bacterial cell contents and culture media in colony PCR reactions often results in polymerase inhibition. A robust polymerase is required to perform colony PCR with high efficiency in many different bacterial strains.

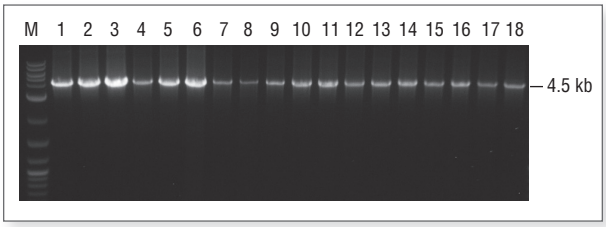
One*Taq* DNA Polymerase, an optimized blend of *Taq* and Deep Vent$_R$™ DNA polymerases, has been formulated for robust yields with minimal optimization. This robustness makes One*Taq* ideal for use in demanding applications, such as colony PCR. (For more information on One*Taq* DNA Polymerase, visit www.neb.com/One*Taq*)

Furthermore, the One*Taq* Quick-Load Master Mix product format increases the ease-of-use for colony PCR. The master mix formulation contains dNTPs, $MgCl_2$, buffer components and stabilizers, as well as two commonly-used tracking dyes for DNA gels. On a 1% agarose gel in 1X TBE, xylene cyanol FF migrates at ~4 kb and tartrazine migrates at ~10 bp. Both dyes are present in concentrations that do not mask any co-migrating DNA bands.

Results

Transformation of a plasmid containing a 4.5 kb insert into eighteen different competent cell lines was performed, followed by colony PCR using either the One*Taq* or One*Taq* Hot Start Quick-Load 2X Master Mix with Standard Buffer. Similar results were obtained using One*Taq* and One*Taq* Hot Start (One*Taq* data not shown), and the 4.5 kb insert was successfully amplified in each case.

Colony PCR of a 4.5 kb insert using One*Taq* Hot Start Quick-Load 2X Master Mix and 18 different *E. coli* strains



Reactions were set up according to the protocol and analyzed by agarose electrophoresis. Marker M is the 1 kb DNA Ladder (NEB #N3232S/L)

Lane	Name	NEB #	Lane	Name	NEB #	Lane	Name	NEB #
1	NEB 10-beta	C3019	7	Lemo21(DE3)	C2528	13	T7 Express lysY	C3010
2	NEB 5-alpha	C2987	8	NiCo21(DE3)	C2529	14	T7 Express	C2566
3	NEB 5-alpha F´Iq	C2992	9	NEB Express Iq	C3037	15	T7 Express Crystal	C3022
4	dam-/dcm-	C2925	10	NEB Express	C2523	16	SHuffle® Express	C3028
5	NEB Turbo	C2984	11	T7 Express Iq	C3016	17	SHuffle T7 Express lysY	C3030
6	BL21(DE3)	C2527	12	T7 Express lysY/Iq	C3013	18	SHuffle T7 Express	C3029

Summary

One*Taq* and One*Taq* Hot Start Quick-Load Master Mixes provide reliable performance in colony PCR, and are compatible with multiple *E. coli* strains. Reliable performance has been seen with amplicons up to 10 kb (data not shown). The Quick-Load format offers additional convenience by enabling direct loading of the PCR reaction onto an agarose gel for analysis. Lastly, the Hot Start formulation provides additional functionality by reducing interference from primer-dimers and secondary amplification products.

GENERAL PROTOCOL

1. Transform ligation mix or other plasmid-containing reaction mixture into the desired bacterial strain, and incubate agar plates overnight at the appropriate temperature.

2. Set up 50 µl reactions as follows:

One*Taq* Master Mix	25 µl
PCR primer	200 nM
Nuclease-free water	to 50 µl

Note: If OneTaq Hot Start Quick-Load 2X Master Mix is used, reactions can be set up at room temperature. If OneTaq Quick-Load 2X Master Mix is used, reactions should be set up on ice.

3. Use a sterile toothpick to pick up individual colonies and dip into each reaction tube.

4. As soon as the solution looks cloudy, remove the toothpick. To create a stock of each individual colony either:

   Dip the toothpick into 3 ml growth media with appropriate antibiotics and culture overnight.

   or

   Streak the toothpick onto another agar plate containing the appropriate antibiotics and grow overnight.

5. Transfer reactions to a thermocycler, and perform PCR following the guidelines below for cycling conditions:

INITIAL DENATURATION	
94°C	2 minutes
30 CYCLES	
94°C	15–30 seconds
45–68°C	15–60 seconds
68°C	1 minute per kb
FINAL HOLD	
68°C	5–10 minutes
10°C	hold

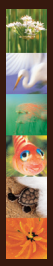
6. Load 4–6 µl of each PCR reaction directly onto an agarose gel, alongside an appropriate DNA ladder.

Note: *The full Application Note can be downloaded at www.neb.com/OneTaq*

NEW ENGLAND BioLabs® Inc.

New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723

Campus-Class™ Mail
Intra-Mail Network



DNA CLONING
DNA AMPLIFICATION & PCR
EPIGENETICS
RNA ANALYSIS
LIBRARY PREP FOR NEXT GEN SEQUENCING
PROTEIN EXPRESSION & ANALYSIS
CELLULAR ANALYSIS

www.neb.com

USA
New England Biolabs, Inc.
Telephone: (978) 927-5054
Toll Free: (U.S. Orders) 1-800-632-5227
Toll Free: (U.S. Tech) 1-800-632-7799
Fax: (978) 921-1350
info@neb.com

Canada
New England Biolabs, Ltd.
Toll Free: 1-800-387-1095
info.ca@neb.com

China, People's Republic
New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
info@neb-china.com

France
New England Biolabs France
Telephone : 0800 100 632
info.fr@neb.com

Germany & Austria
New England Biolabs GmbH
Free Call 0800/246 5227 (Germany)
Free Call 00800/246 52277 (Austria)
info.de@neb.com

Japan
New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
info@neb-japan.com

Singapore
New England Biolabs Pte. Ltd.
Telephone +65 6776 0903
sales.sg@neb.com

United Kingdom
New England Biolabs (UK), Ltd.
Call Free 0800 318486
info.uk@neb.com

Do you use NEB's interactive tools?

Our popular interactive tools for research and experimental design are available at www.neb.com/tools-and-resources/interactive-tools

Double Digest Finder and Enzyme Finder are also featured on NEBTools, our Free App for iPhone® and Android™.