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NEB expressions

a scientific update

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New England Biolabs, Inc.

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in this issue

Substrate specificity and mismatch discrimination in DNA ligases

DNA ligases vary in their ability to join fragments, add adaptors, repair nicks or breaks, link vectors and inserts, and to circularize dsDNA. Ligases also vary in their type of activity. The specificity and accuracy of the ligation depends upon ligase selection and careful optimization of reaction conditions. With the right ligase, conditions and probes, even single-base variations in sequence can be reliably detected.

DNA ligases are enzymes that seal breaks in DNA by joining 5′-phosphorylated DNA termini to 3′-OH DNA termini (1-4). *In vivo*, ligases are important for the repair of nicks, breaks in one strand of a dsDNA molecule, DNA formed during replication (i.e., Okazaki fragments), as well as both nick and double-strand break joining during repair events (5). *In vitro*, ligases (notably T4 DNA Ligase) are critical reagents for many molecular biology protocols, including vector-insert joining for recombinant plasmid construction, adaptor ligation for next-generation sequencing (NGS) library construction, and circularization of dsDNA (6). T4 DNA Ligase (NEB #M0202) is incredibly efficient at sealing nicks, as well as joining or circularizing DNA fragments with blunt or cohesive (short complementary) ends. This activity can be further improved with the addition of macromolecular enhancers, such as polyethylene glycol (PEG), as seen in NEB's Quick Ligation™ Kit (NEB #M2200) (7,8).

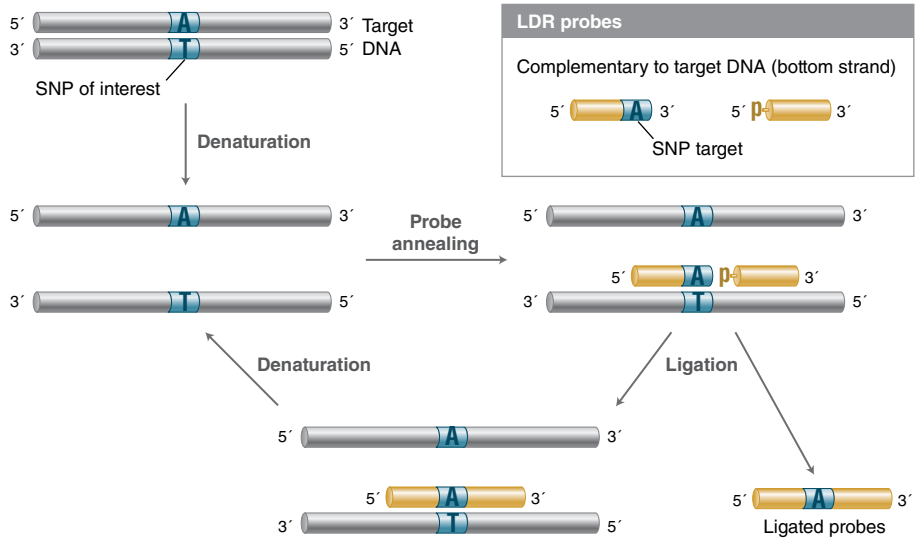
Less commonly utilized *in vitro*, *Taq* DNA Ligase (NEB #M0208) will ligate only nicks (9-12). *Taq* Ligase is a NAD^+-dependent DNA ligase from a thermostable bacterium that can survive high temperatures (up to 95°C) and is active over a range of elevated temperatures (37–75°C). However, it only has significant activity on nicked DNA, and negligible activity on short cohesive and blunt substrates in end-joining reactions. Given these limitations, and the fact that T4 DNA Ligase can ligate everything *Taq* ligates, and many more structures, why not use T4 DNA Ligase for all applications?

T4 DNA Ligase can ligate a wide variety of DNA structures, including modified bases and the ends of double stranded fragments. It will also efficiently ligate many undesirable structures, including substrates containing gaps of one or more nucleotides and nicked substrates that contain DNA base pair mismatches (12-15). In most cases, this unwanted activity isn't a problem, for example, when joining 1 or 2 fragments into a plasmid, or pushing an adaptor ligation reaction as far towards completion as possible to prepare high yields of DNA NGS libraries.

For some applications, however, there cannot be any end-joining activity at all, and for others, there is a need for the exclusive ligation of fully base-paired nicks with no gaps. For example, DNA assembly methods, such as Gibson Assembly® (NEB #E5510) and NEBuilder® HiFi DNA Assembly (NEB #E2621), require nick-selective ligases; this method utilizes long overlaps that are dynamically generated by exonucleases and gaps are filled by a DNA polymerase (16,17). Final joining is accomplished by a nick-selective ligase, such as *Taq* DNA Ligase, which only reacts with substrates containing no gaps, and will not join any fragments end-to-end without the exo/polymerase generation of annealed complementary regions. The use of a nick-selective ligase ensures that fragments are not joined out of order, and no deletions result from ligation across nucleotide gaps in annealed structures. (For more information, see www.neb.com/DNAassembly).

FIGURE 1:
Ligase Detection Reaction (LDR)

Two ligation probes are designed such that they are complementary to a target region of interest, and anneal with no gaps. Typically, if a SNP is to be resolved, the nucleotide of interest is situated at the junction of the two probes. The probes are combined with the DNA to be examined (typically genomic or PCR amplified region) and the thermostable DNA ligase. The DNA is melted, then cooled to a ligation temperature that allows the probes to anneal to the target. If the probes anneal to form a nicked structure with no gaps or mismatches, efficient ligation will proceed. Cycling, melting and annealing/ligation allows successive rounds of probes to anneal and ligate, resulting in linear amplification of the ligation product if the sequence of interest is present in the target DNA.



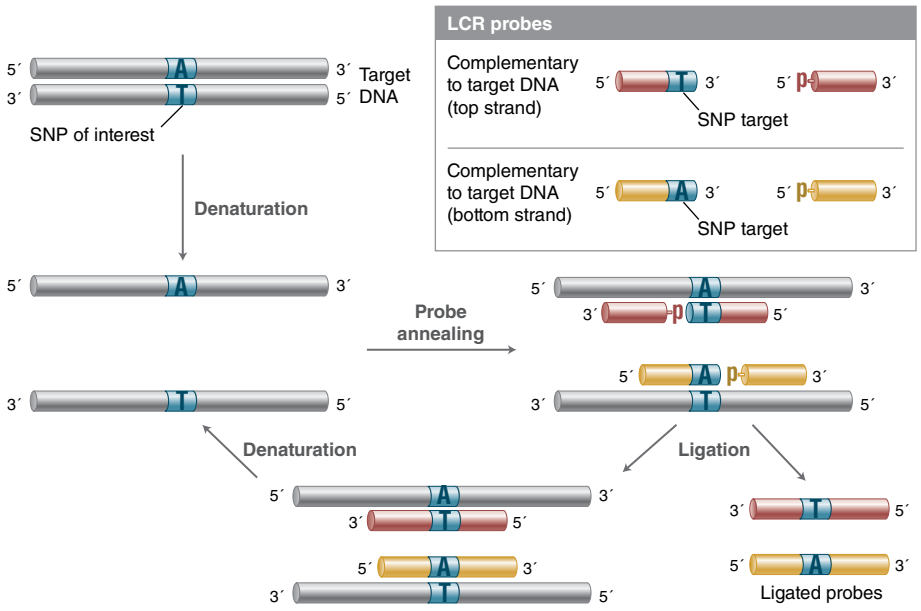
LIGASE SPECIFICITY

DNA ligases generally prefer fully Watson-Crick base-paired dsDNA substrates to those containing one or more mismatches. However, ligases can ligate some mismatches to a significant degree, and very active ligases, such as T4 DNA Ligase, can ligate nicks containing one or more mismatches near the ligation junction with high efficiency (15,18). Ligases are thought to interrogate dsDNA for proper base pairing through minor grove contacts, and thus do not read specific base sequences, but are sensitive to distortions in helix shape (19). Large purine:purine mismatches and most smaller pyrimidine:pyrimidine mismatches are typically worse ligation substrates than pyrimidine:purine mismatches. Helix stability also plays some role, and mismatches with more hydrogen bonds are more readily ligated than those with few. For many ligases, G:T mismatches, with two hydrogen bonds and a base-pair size nearly indistinguishable from a Watson-Crick base pair, are joined with nearly the same efficiency as a correct base pair. Additionally, DNA ligases have been generally found to have a higher discrimination at the upstream side of the ligation junction (the base pair providing the 3′-OH terminus to the ligation) than on the downstream side (the base pair providing the 5′-phosphate to the ligation). The structural/mechanistic reason for this differential is not known for certain, but may have to do with the slight melting of the 5′-terminus during the reaction. This "peeling back" of the 5′-phosphorylated base can be observed in the crystal structures of several DNA ligases bound to substrate (20,21).

Thermostable DNA ligases, including *Taq* DNA ligase, are naturally able to discriminate against

FIGURE 2:
Ligase Chain Reaction (LCR)

In this method, two pairs of probes are designed, one pair complementary to the top strand of the target, one pair to the bottom. Upon melting and annealing, both probe pairs can anneal to the target, and ligate efficiently if they form a nicked sequence without gaps or mismatches. On successive rounds of melting and re-annealing, unligated probe can now anneal to both the original target DNA and to the probes ligated in previous rounds. As each ligation product becomes a template for the complementary probe pair, LCR enables exponential amplification of the ligated product.



ligating substrates containing base pair mismatches (i.e., are "higher fidelity") than T4 DNA Ligase (18,22,23). Despite this higher fidelity, *Taq* DNA Ligase can still detectably ligate many T:G, T:T, and A:C mismatches. Thermostable DNA ligases are active at elevated temperatures, allowing further discrimination by incubating the ligation at a temperature near the melting temperature (Tm) of the DNA strands. This selectively reduces the concentration of annealed mismatched substrates (expected to have a slightly lower Tm around the mismatch) over annealed fully base-paired substrates. Thus, high-fidelity ligation can be achieved through a combination of the intrinsic selectivity of the ligase active site and careful balance of reaction conditions to reduce the incidence of annealed mismatched dsDNA.

APPLICATIONS REQUIRING HIGH-FIDELITY LIGATION

Numerous applications have been developed that take advantage of the high fidelity of *Taq* and other thermostable DNA ligases to detect specific nucleotide sequences with high specificity and quantitative accuracy, including profiling single nucleotide polymorphisms (SNPs) (9,24,25). In the Ligase Detection Reaction (LDR), a set of probes complementary to the sequence of interest are annealed to target DNA (genomic DNA, or a PCR amplified fragment) and treated with a high-fidelity thermostable DNA ligase (Figure 1, page 2). If the target sequence is present, the probes will ligate; cycling through rounds of melting and annealing can allow linear amplification of the probe ligation product. With the right ligase, conditions and suitable probes, single-base differences can be reliably detected. The original paper detected the ligation product through visualization in a gel, but detection through fluorophore-quencher pairs or qPCR-based methods can greatly increase the sensitivity of detection (26-32). LDR has also been extended to multiplexed probe sets that allow the simultaneous interrogation of multiple potential SNP sites (27).

The closely related Ligase Chain Reaction (LCR) takes the LDR method and makes it amplifiable in an exponential fashion (9,24). In LCR, four probes are used, one pair complementary to one target strand, and a second pair of probes complementary to the other strand (Figure 2). Since the probe pairs are complementary to each other, the probe ligated in one cycle becomes a template for ligation of additional probe in subsequent cycles. This methodology allows for detection of SNPs with greater sensitivity than the original LDR method, but requires extraordinary discrimination against mismatch ligation for both probe sets, as even trace ligation on a mismatched template will result in template for further probe amplification (and thus, an erroneous positive signal). The complementarity of LCR probe pairs also means that probes can anneal to each other, forming blunt-end or single-base overhang substrates, depending on probe design strategy. While most high-fidelity ligases have far lower activity on double-stranded fragment end joining, even trace blunt-end activity will generate template for further rounds of high-efficiency nick ligation. Thus, LCR can suffer from high non-templated background as well, and requires careful probe design. The modification gap-LCR method attempts to address these background and discrimination issues by utilizing probes which anneal with a single-nucleotide gap that must be filled by a polymerase in order to generate a substrate suitable for ligation (33). This modification leverages the discrimination against gap ligation of thermostable high-fidelity ligases, but requires a thermostable polymerase and dNTPs as well.

FIGURE 3:
Ligation-Rolling Circle Amplification/Padlock Probes

In this method, a single probe is designed such that the ends of the probes are complementary to the target sequence. When annealed to the desired target, the ends form a nicked structure that can be efficiently ligated if there are no gaps or mismatched base pairs. Exonuclease treatment destroys the uncircularized DNA, and the remaining circular structures can be detected through rolling circle amplification (RCA), or linearized and amplified with PCR.

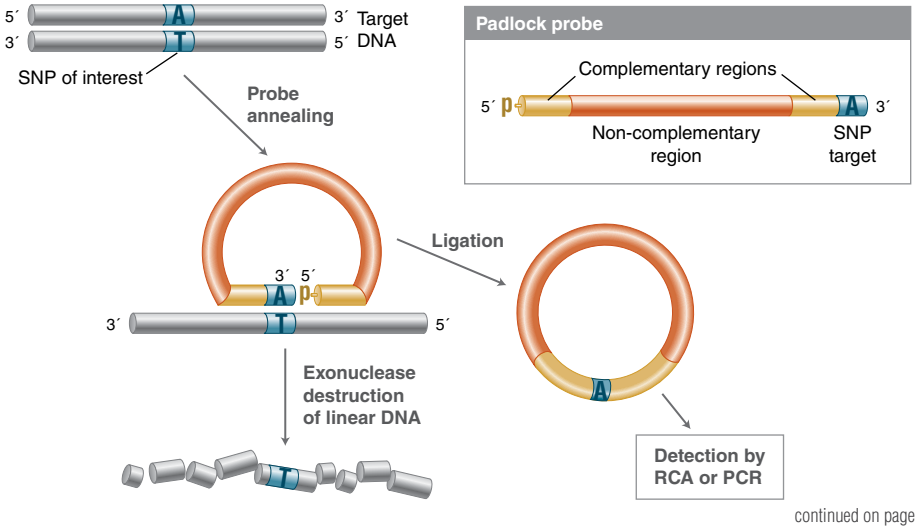


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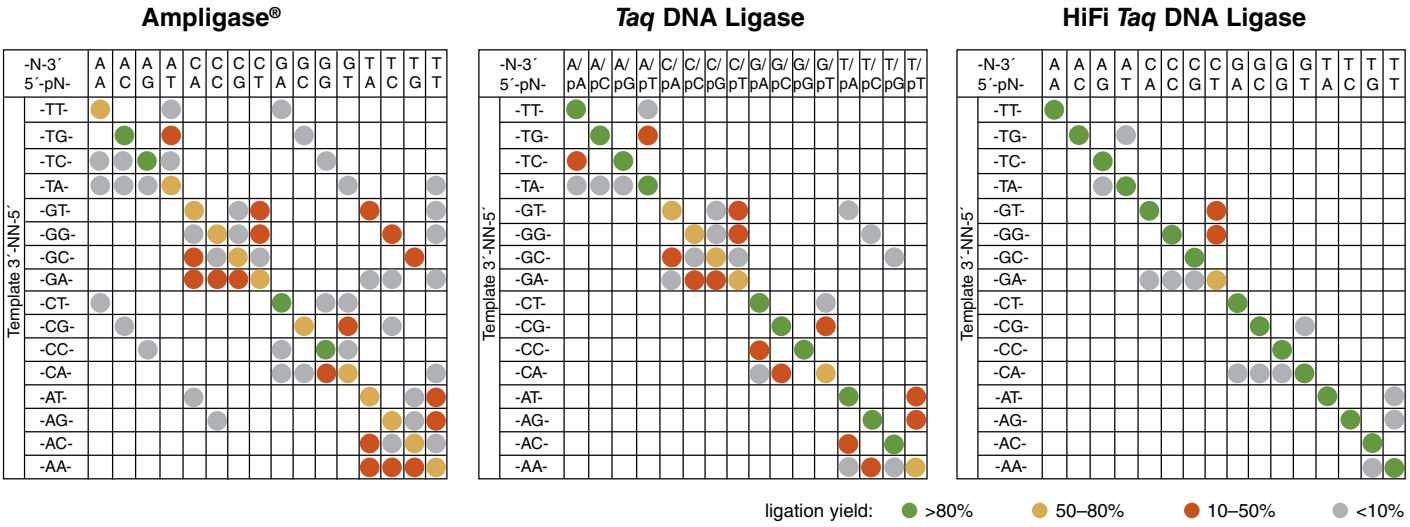
FIGURE 4:
Comparison of fidelity of *Taq* DNA Ligase (NEB #M0208), Ampligase (Epicentre), and HiFi *Taq* DNA Ligase (NEB #M0647)

Fidelity measurements were performed using 1 µL of ligase in a 50 µL reaction mixture in the supplied buffers at 1x concentration. Reactions were incubated 30 min at 55°C, using multiplexed substrate pools as outlined in our previous publication (Lohman, G.J. et al. 2016). The rows represent a single template sequence, while columns indicate a particular ligation product resulting from a specific pair of probes ligating with the indicated bases at the ligation junction. A dot indicates detection of a product (see legend). The diagonal from the top left to the bottom right represents Watson-Crick ligation products; all other spaces indicate mismatch ligation products. While *Taq* ligase and AmpLigase perform similarly under these conditions, with a range of mismatch products detectable, HiFi *Taq* Ligase shows dramatically fewer mismatch products while maintaining high yields (image adpated from Lohman, G.J. et al. 2016).



Additional detection-by-ligation technologies have been devised to take advantage of high-fidelity ligation events by generating circular templates that can be detected in a secondary reaction (34-38). In the "padlock" probe design, a single-stranded probe is devised where the 5´ and 3´ ends are both complementary to a target sequence (Figure 3, page 3). Much like LDR, the ends of this single probe form a nick structure with no gaps when annealed, and can only be ligated when fully base-paired to a complementary target. All single-stranded DNA can be destroyed by exonuclease treatment, and the circularized probes can be detected by methods such as rolling circle amplification, or linearized and detected by PCR. Variation on the padlock probe design include "molecular inversion probes," which, similar to gap-LCR, have a single-stranded probe in which both ends are annealed with a gap of one or more nucleotides that must be filled by a polymerase before ligation can occur.

OTHER FACTORS TO CONSIDER

These and other detection-by-ligation methodologies depend on the ability of the ligase to discriminate against substrates containing one or more mismatches, yet retain high activity on even low concentrations of the fully base-paired probe-target structure. While the choice of ligase is very important, careful probe design, selection of reaction temperature, and even ligation buffer conditions can all contribute to the fidelity of the ligation reaction, and thus the accuracy and sensitivity of the detection-by-ligation. For example, probes should take advantage of the naturally higher discrimination of ligases on the upstream side of the ligation junction (the base pair providing the 3´-hydroxyl). Probes that place the base of interest on the downstream side will provide significantly poorer discrimination, and probes will ligate on templates containing other bases at the targeted position. Furthermore, it is important to know what base pair mismatches are more easily ligated by a given ligase. For example, if you are targeting a position that can be an A:T or a T:A base pair, it would be better to use a probe with an A at the 3´ end (targeting the strand with a T at the SNP position) than to use a probe with a T; when annealing to the wrong SNP, the first case would result in a difficult-to-ligate A:A mismatch, while the second would result in a T:T mismatch that can be ligated by *Taq* DNA Ligase with relatively high efficiency.

Incubation temperature is also a key consideration, and typically must be optimized for each application. If the ligation temperature is too far below the Tm of the probes, even mismatched probes will be annealed, increasing the chances of ligation occurring. If the ligation temperature is too far above the Tm, fully complementary sequences will not be annealed. High-fidelity ligation reactions should typically be run 1–2°C below the Tm of the probes to give the highest possible accuracy by minimizing the concentration of annealed mismatched probes. Consequently, it is important to match the Tm of both the upstream and downstream probe annealing regions, and all probe sets when attempting a multiplexed reaction. If there is a range of Tm values for the probe annealing regions, no single reaction temperature will result in the optimal balance of fidelity versus activity for all probe sets.

Buffer conditions can also affect the fidelity of DNA ligases. In particular, it has been observed for several thermostable DNA ligases, including T4 DNA Ligase and Human DNA Ligase 3, that increasing monovalent cation concentration improves the fidelity of ligation (15,39) . This effect is thought to be related to a weakening of the binding of the ligase to its substrate, with a disproportionate suppression binding/ligation of mismatched substrates. Too much salt can erase activity on even fully base-paired substrates, and the best salt balance for each ligase must be empirically determined.

OPTIMIZATION THROUGH HIGH-THROUGHPUT PROFILING

NEB researchers recently published a method for the high-throughput profiling of ligase fidelity, a method that extends earlier studies through a high-sensitivity multiplexed format (18,40). This methodology has been used to rapidly screen buffer conditions for *Taq* DNA Ligase and their effect on fidelity. In this method, substrate pools were prepared consisting of one target (template) strand and four upstream probes and four downstream probes, each differing only in the base at the ligation junction. Thus, all four bases at either side of the ligation junction were represented. Sixteen separate pools were prepared, each with a different template strand covering all 16 possible NN pairs in the template as well. The probes were designed such that each possible pairing resulted in a product of unique length, with products repeatable and quantifiable by capillary electrophoresis (CE). This method allowed screening of all possible base combina-

tions (Watson-Crick and mismatched) around the ligation junction in 16 wells of a 96-well plate, allowing 6 conditions to be screened per plate. The results indicated that the optimal buffer for *Taq* DNA Ligase contains with 100 – 200 mM KCl at pH 8.5.

CONCLUSION

This optimization method has been used internally at NEB to screen additional ligases, conditions and formulations, and has led to the development of the new HiFi *Taq* DNA Ligase (NEB #M0647, see below). Using this method, both the enzyme and the reaction buffer were optimized, resulting in the highest fidelity NAD^+-dependent DNA ligase commercially available (Figure 4).

It is important to note that thermostable, high-fidelity, nick-selective DNA ligases like *Taq* DNA Ligase, HiFi *Taq* DNA Ligase, and the ATP dependent 9°N™ DNA Ligase (NEB #M0238), are not replacements for T4 DNA Ligase in applications such as routine cloning or DNA library preparation. However, when a method relies on accurate ligation of nicks lacking gaps or mismatched base pairs, using of one of these ligases, combined with careful probe design and reaction condition optimization, will be critical for success.

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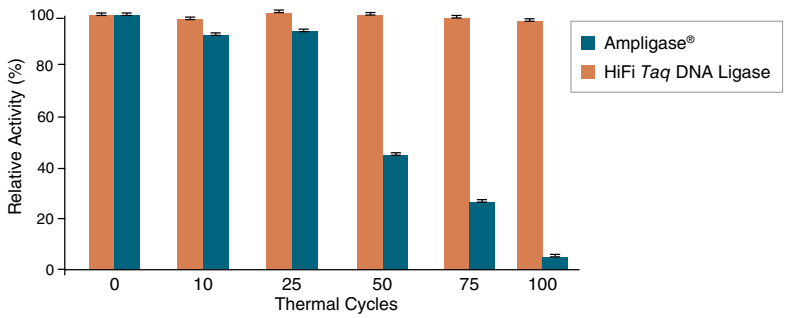
NEW PRODUCT

HiFi *Taq* DNA Ligase

Optimized HiFi *Taq* DNA Ligase efficiently seals nicks in DNA with unmatched fidelity (See Figure 4, page 4). Ligation of two adjacent oligonucleotides that are hybridized to a complementary target DNA is enhanced by an improved reaction buffer, and mismatch ligation is dramatically reduced. Furthermore, HiFi *Taq* DNA Ligase exhibits increased thermostability and is active at elevated temperatures (37–75°C) for extended cycles. This improved formulation allows for higher resolution discrimination between ligation donors and acceptors at both the 3´ and 5´side of the ligation junction, enabling precise detection of SNPs and other allele variants via detection methods such as LCR and LDR.

HiFi *Taq* DNA Ligase exhibits increased thermostability

HiFi *Taq* DNA Ligase and Ampligase® (1 µL enzyme in a 50 µL reaction) were cycled (80°C for 90 seconds/94°C for 10 seconds) up to 100 times in their respective 1X reaction buffer. Ligase activity was assayed using a FAM-labeled nicked dsDNA substrate detected by capillary electrophoresis.



advantages

- High fidelity
- Increased thermostability (as compared to Ampligase®)
- Recombinant source
- Improved discrimination at both the 3´ and 5´side of the ligation junction
- Online tool to calculate optimal ligation temperature (ligasecalc.neb.com)

ORDERING INFORMATION:

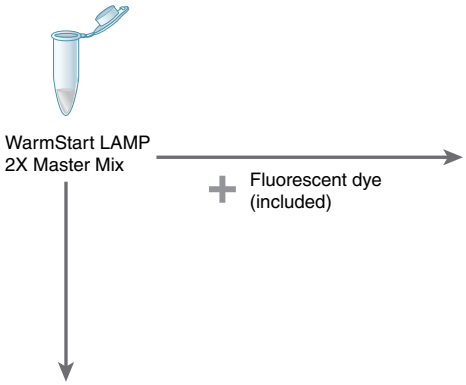
PRODUCT	NEB #	SIZE
HiFi *Taq* DNA Ligase	M0647S	50 rxns

New Products for Isothermal Amplification

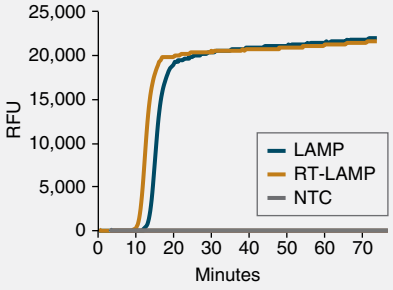
WarmStart® LAMP Kit (DNA & RNA)

Loop Mediated Isothermal Amplification (LAMP) is a commonly-used technique for rapid nucleic acid detection. NEB's WarmStart LAMP products provide a simple, one-step solution for DNA or RNA targets. The master mix supplied with the WarmStart LAMP Kit contains the robust and rapid Bst 2.0 WarmStart DNA Polymerase and WarmStart RTx Reverse Transcriptase, both *in silico*-designed enzymes for improved performance in LAMP reactions. The kit also includes a fluorescent dye to enable real-time fluorescence measurement of LAMP. The WarmStart LAMP Kit is compatible with multiple detection methods, as shown in the figure below.

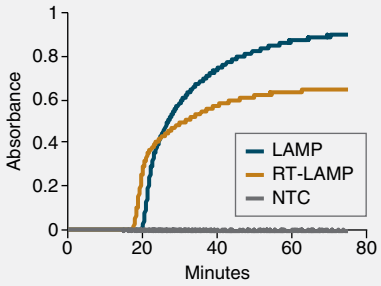
NEB's WarmStart LAMP Kit (DNA & RNA) is compatible with multiple detection methods*

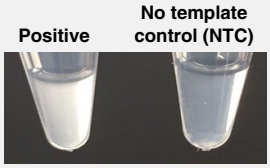


Real-time fluorescence measurement



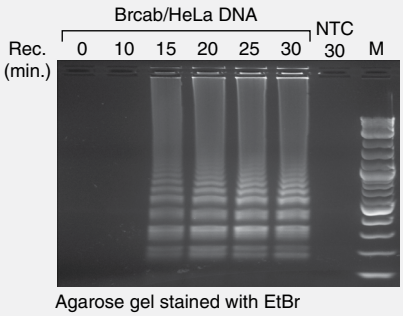
Turbidity detection



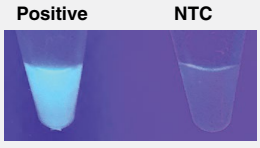


LAMP product is visible by eye due to the precipitation of magnesium pyrophosphate

Endpoint visualization



Agarose gel stained with EtBr



Visualization using a fluorescent intercalating dye (e.g., ethidium bromide, SYBR® green or calcein/HNBlue)

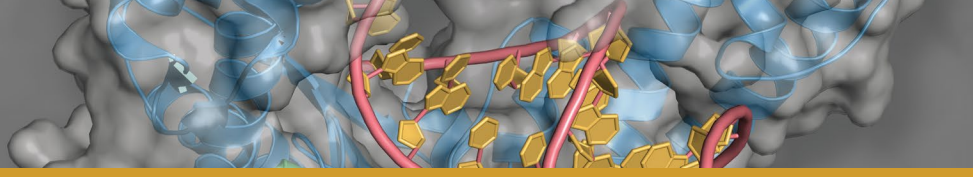
** The NEB WarmStart LAMP Kit (DNA & RNA) includes separate fluorescent dye for real-time fluorescence measurement. Alternately, detection can be accomplished by turbidity detection or endpoint visualization.*

looking for best-in-class visual detection?

Try our WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA)

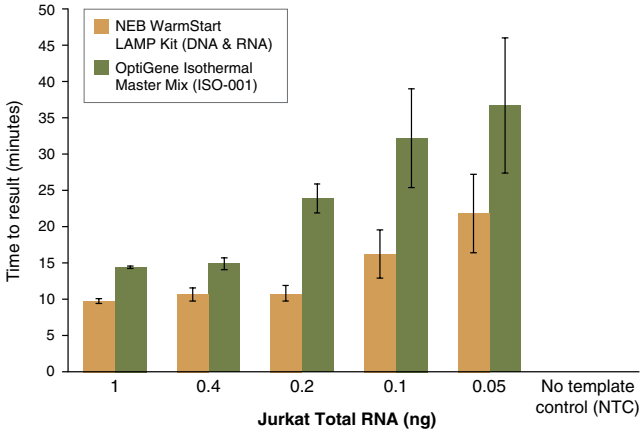
- NEB's WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA) offers the same robust performance as the WarmStart LAMP Kit, and contains a colorimetric dye for best in class visual detection of your target.

Yellow color indicates that amplification has occurred.



NEB's WarmStart LAMP Kit (DNA & RNA) offers speed and robust sensitivity

A RNA target (HMBS2) was amplified from Jurkat total RNA using the WarmStart LAMP Kit and OptiGene Master Mix (ISO-001). Reactions were performed at 65°C for 74 minutes on a real-time thermocycler (Bio-Rad® CFX96) in triplicate. Time to result is set as the time at which the fluorescence crossed a threshold of 10% of maximal fluorescence. NEB's WarmStart LAMP Kit resulted in faster and more sensitive detection as compared to the OptiGene Master Mix.



ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
WarmStart LAMP KIT (DNA & RNA)	E1700S/L	1,600/8,000 units
WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA)	M1800S/L	100/500 rxns
Bst 2.0 WarmStart DNA Polymerase	M0538S/M/L	1,600/8,000 units
WarmStart RTx Reverse Transcriptase	M0380S/L	50/250 rxns

optimization tips for LAMP

- Use LAMP primer design software (e.g., Primer Explorer – primerexplorer.jp/e/). Select 2–3 sets for each target and compare performance in a LAMP assay.
- Include loop primers for faster reactions
- Use high magnesium (6–8 mM) and dNTP (1–1.4 mM) concentrations for best results
- Omit betaine, unless it has a demonstrated benefit
- Optimize the reaction temperature (60–65°C for Bst LF and 63–70°C for Bst 2.0/3.0)
- To prevent contamination, use Bst 3.0 or Antarctic Thermolabile UDG (NEB #M0372), which denatures rapidly

Did you know?

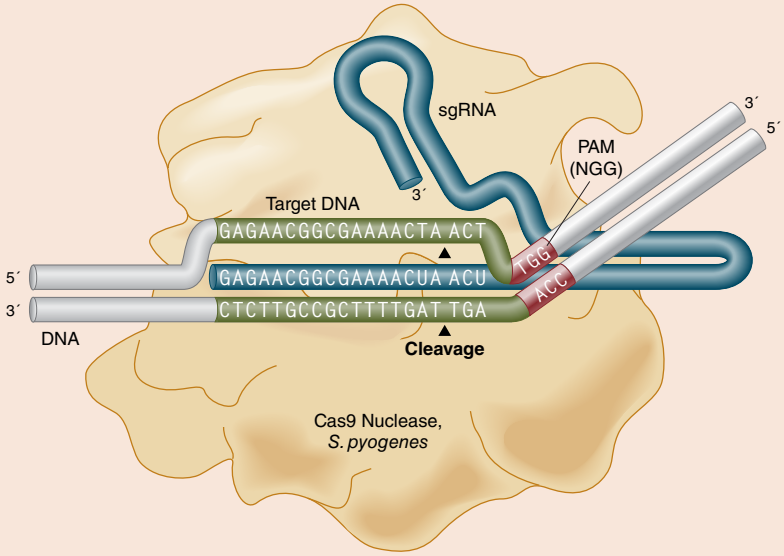
All of the wastewater from our Ipswich, MA campus is treated by a state-of-the-art Solar Aquatics® System. This system utilizes and accelerates the natural purification processes found in streams and wetlands, purifying the water for reuse or for groundwater recharge.

Learn more and take a tour of the facility at www.neb.com/environmentalphilosophy

Tools to support your genome editing workflows

New England Biolabs provides reagents to support a broad variety of CRISPR/Cas9 genome editing approaches. From introduction of Cas9 and single guide RNA (sgRNA) on plasmids, to direct introduction of Cas9 ribonucleoprotein (RNP) and detection of edits using next generation sequencing or enzymatic mutation detection, NEB provides reagents that simplify and shorten genome editing workflows. Generating RNPs for direct introduction requires Cas9 protein and either sgRNA or separate crRNA and tracrRNA. EnGen® Cas9 NLS, *S. pyogenes* is engineered for high genome editing efficiency. The EnGen sgRNA Synthesis Kit combines template assembly and *in vitro* transcription for rapid generation of microgram quantities of custom sgRNA, requiring only a user-supplied single ssDNA oligonucleotide. To determine editing efficiency, the EnGen Mutation Detection Kit provides a full workflow from PCR amplification to T7 Endonuclease I-based mutation detection. Alternatively, NEB supplies Cas9 wild type and restriction enzymes, both of which can be used *in vitro* to determine the extent of editing.

Schematic representation of Cas9 Nuclease, *S. pyogenes* sequence recognition and DNA cleavage

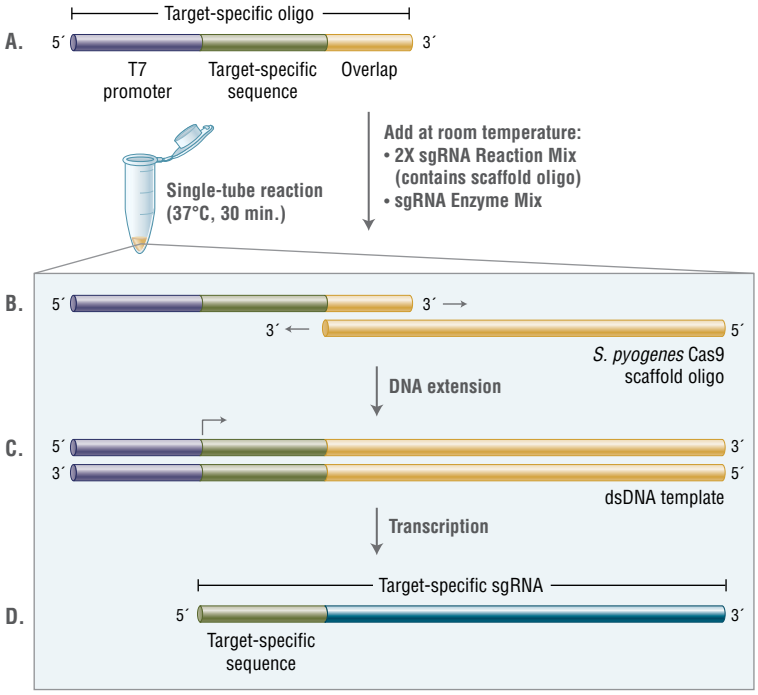


Visit **www.neb.com/GenomeEditing** to view our online tutorial which walks through genome editing with Cas9

EnGen sgRNA Synthesis Kit

The EnGen sgRNA Synthesis Kit simplifies the generation of microgram quantities of custom sgRNAs in an hour or less by combining template synthesis and transcription. The single-tube reaction is easy to set up and requires a single ~55 nt ssDNA target-specific oligonucleotide, which is combined with the Reaction Mix and Enzyme Mix included in the kit. sgRNAs are suitable for use in downstream applications, including CRISPR/Cas9-based genome editing and *in vitro* DNA cleavage. This single-reaction format offers ease-of-use and eliminates separate DNA amplification and template clean up steps.

EnGen sgRNA Synthesis Kit overview



"This kit is really easy to use and will save us plenty of time in making sgRNAs! Thanks for the streamlined method!"

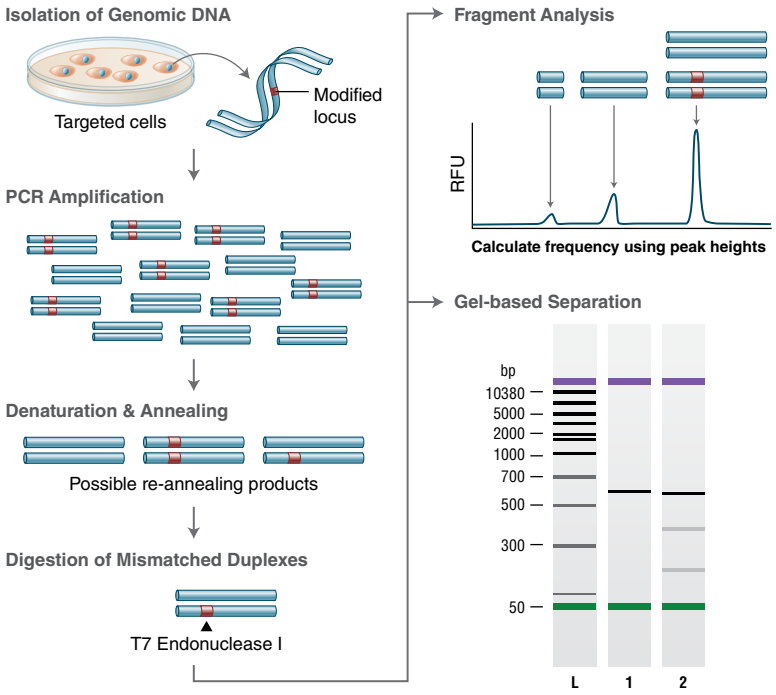
– Postdoctoral Researcher,
Harvard University

EnGen Mutation Detection Kit

A widely used method to identify mutations is the T7 Endonuclease I mutation detection assay. This assay detects heteroduplex DNA that results from the annealing of a DNA strand, including desired mutations, with a wild-type DNA strand. The EnGen Mutation Detection Kit provides optimized reagents for performing robust T7 Endonuclease-based detection of genome editing events.

Workflow for EnGen Mutation Detection Kit

Genomic DNA is amplified with primers bracketing the modified locus. PCR products are then denatured and re-annealed yielding three classes of possible structures. Duplexes containing a mismatch greater than one base are digested by T7 Endonuclease I. The DNA is then electrophoretically separated and fragment analysis is used to estimate targeting efficiency.



ORDERING INFORMATION:

PRODUCT	NEB #	SIZE
EnGen sgRNA Synthesis Kit , *S. pyogenes*	E3322S	20 rxns
EnGen Mutation Detection Kit	E3321S	25 rxns
EnGen Cas9 NLS, *S. pyogenes*	M0646T/M	400/2,000 pmol
T7 Endonuclease I	M0302S/L	250/1,250 units
Q5® Hot Start High-Fidelity 2X Master Mix	M0494S/L	100/500 rxns

online resources

Plasmid Repositories
- addgene.org

CRISPR-gRNA Design Tools
- deskgen.com
- crispr.mit.edu
- zifit.partners.org/ZiFiT
- e-crisp.org
- chopchop.rc.fas.harvard.edu
- benchling.com

Online Forums
- groups.google.com/forum/#!forum/crispr

Organism-specific Resources
- wormcas9hr.weebly.com
- flyrnai.org

Visit www.neb.com/GenomeEditing to find:

Protocols for applications such as sgRNA synthesis and direct introduction of sgRNA/Cas9 complexes

EnGen sgRNA Template Oligo Designer (accessible through NEBioCalculator® at **NEBiocalculator.neb.com**)

Our latest **Genome Editing Brochure** available for download

Construction of an sgRNA-Cas9 expression vector via single-stranded DNA oligo bridging of double-stranded DNA fragments

Peichung Hsieh, Ph.D., New England Biolabs, Inc

INTRODUCTION

NEBuilder® HiFi DNA Assembly Master Mix (NEB# E2621) was developed to improve the efficiency and accuracy of DNA assembly over other DNA assembly products currently available. The method allows for the seamless assembly of multiple DNA fragments, regardless of fragment length or end compatibility. Thus far, it has been adopted by the synthetic biology community, as well as those interested in one-step cloning of multiple fragments, due to its ease of use, flexibility and simple master mix format.

CRISPR/Cas9-based approaches are quickly growing in popularity in the field of genome editing. Due to the size of most commonly used Cas9-containing plasmids, construction of an sgRNA or sgRNA library into a Cas9/sgRNA expression vector can be cumbersome. NEB has developed a protocol to solve this problem using single-stranded DNA oligonucleotides, a restriction enzyme digested vector and the NEBuilder HiFi DNA Assembly Master Mix.

PROTOCOL

1. Scan for a PAM sequence (NGG, in green) in the desired target sequence.

   Example:
   5´GCGAAGAACCTCTTCCCAAGANGG3´

   We suggest using the sgRNA design tools available at:
   https://chopchop.rc.fas.harvard.edu
   https://www.deskgen.com
   https://benchling.com

2. Design a 71-base, single-stranded DNA oligonucleotide, containing a 21 nt target sequence (in red), flanked by a partial U6 promoter sequence (in blue) and scaffold RNA sequence (in purple).

   Example:
   5´ATCTTGTGGAAAGGACGAAACACCG
   GCGAAGAACCTCTTCCCAAGA
   GTTTTAGAGCTAGAAATAGCAAGTT3´

   To construct a random library, randomize nucleotides 19–21, as shown below:

   Example:
   5´ATCTTGTGGAAAGGACGAAACACCG
   N_{19-21}GTTTTAGAGCTAGAAATAGCAAGTT3´

3. Prepare the ssDNA oligo in 1X NEBuffer 2 to a final concentration of 0.2 µM.
4. Assemble a 10 µl reaction mix with 5 µl of ssDNA oligo (0.2 µM), 30 ng of restriction enzyme-linearized vector* and ddH_2O.
5. Add 10 µl of NEBuilder HiFi DNA Assembly Master Mix to the reaction mix, and incubate the assembly reaction for 1 hour at 50°C.
6. Transform NEB 10-beta Competent *E. coli* (NEB #C3019) with 2 µl of the assembled product, following the protocol supplied with the cells.
7. Spread 100 µl of outgrowth on a plate with ampicillin antibiotic, and incubate overnight at 37°C.
8. Pick 10 colonies to grow, and purify the plasmid DNA for sequencing.

* Researchers can find suitable vectors from Addgene. For example, Addgene plasmid #42230, pX330-U6-Chimeric_BB-CBh-hSpCas9 (for details, see https://www.addgene.org/42230/), although any plasmid containing an sgRNA scaffold under the control of a U6 promotor should be adequate.

RESULTS

Ten colonies were isolated, and plasmid DNA was purified using standard miniprep columns (we recommend Monarch® Plasmid Miniprep Kit, NEB #T1010). Insertion of the target DNA sequence was confirmed by DNA sequencing. Of the 10 clones sequenced, 9 contained the target sequence in the correct orientation (data not shown).

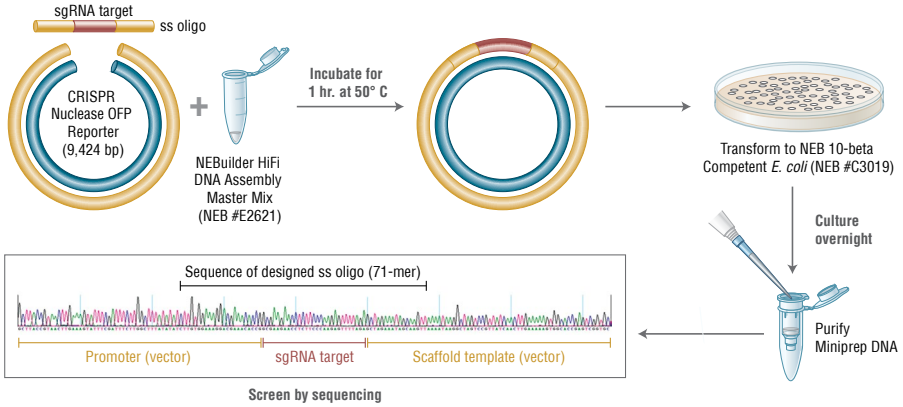
SUMMARY

This Application Note demonstrates the convenience of inserting an sgRNA sequence into a 9.5 kb vector for targeted DNA assembly. Unlike traditional cloning methods, in which two oligos must be synthesized and re-annealed, this new protocol offers a simple way to design an oligo and assemble it with the desired vector.

The NEBuilder HiFi DNA Assembly Master Mix represents a substantial improvement over traditional methods, specifically in time savings, ease-of-use and cost.

sgRNA cloning workflow

Design a ssDNA oligo containing the target sequence (19-21 bases) of sgRNA flanked by 25 bases of sequence at both ends. Mix the single-stranded oligo, linearized vector DNA and NEBuilder HiFi DNA Assembly Master Mix together, incubate for 1 hour at 50°C and transform into *E. coli.*



Interested in learning more?

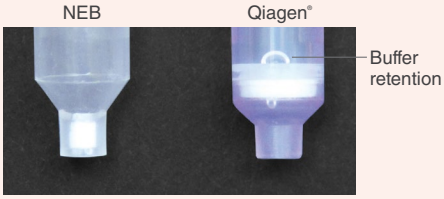
Visit **www.neb.com/E2621** to **download the full application note**, which includes data and tables not shown.

4 Reasons to Migrate to Monarch®

Monarch Nucleic Acid Purification Kits offer fast and reliable purification of high-quality DNA, using best-in-class technology. Here are four reasons that Monarch columns provide superior performance over other commercially-available nucleic acid purification columns.

1 No buffer retention

Many purification columns are built with a frit to hold the membrane in place, and this can trap buffer during various steps in the protocol. With Monarch columns, the silica matrix is held in place without the use of a frit, thereby eliminating buffer retention and the risk of carryover contamination.



2 Low elution volumes

The tapered design of Monarch columns enables elution in as low as 30 µl for the Miniprep Kit and 6 µl for the Gel Extraction and PCR & DNA Cleanup Kits, resulting in highly concentrated DNA for use in downstream applications.

3 Convenience

Monarch columns include convenient labeling areas and fit snugly into the collection tube for easy handling. Monarch columns are suitable for use with centrifugation and vacuum purification protocols and are also available for purchase separately.

4 Less environmental impact

Monarch columns use less plastic than conventional columns, reducing their environmental footprint without compromising performance. You can feel good about choosing Monarch for your DNA purification needs!

See what customers are saying about our Monarch Kits:

Having my isolated DNA in a more concentrated form allows for smoother downstream reactions. (e.g., using only 1 µl instead of 6-12 µl in a ligation reaction gives me more flexibility.)

(I like that you are) able to buy buffers individually, most other kits only give you the minimum amount required (not so good if you have a spill!)

Column eliminates any traces of previous buffers, which is especially important for the final elution step.

I found that being able to elute the DNA in much smaller volumes greatly helped in the subsequent steps.

At least 100 ng/µl higher in concentration!

Monarch Kits are available for Plasmid Miniprep, DNA Gel Extraction, and PCR & DNA Cleanup

Visit **NEBMonarch.com** to learn more and request a sample.

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