Webinar Q&A: NEBuilder HiFi: The Next Generation of DNA Assembly

Q: What is happening during the 1 hour incubation at 50°C?

A: During that 1 hour incubation, T5 Exonuclease is chewing the DNA in the 5´ to 3´ direction to generate the ssDNA overlaps that will allow the DNA fragments to anneal. The polymerase then fills in the gaps in the annealed fragment and the ligase seals those nicks. Here is a quick video illustrating the assembly process.

Q: How long can the 3´ and 5´ mismatches be?

A: We recommend 3´ and 5´ mismatches <10 bp; this works very efficiently. Longer mismatches can be tolerated but the efficiency decreases as the mismatches become longer.

Q: Sometimes I want to cut my plasmid with a single enzyme that cuts twice. Why can't NEBuilder handle this situation?

A: The NEBuilder HiFi DNA Assembly Master Mix can perform this assembly. It doesn't matter that the ends of the plasmid were generated by digestion with one or two enzymes. The NEBuilder Assembly Tool is being configured to handle restriction enzymes that cut more than once in a plasmid. In the meantime, there is a work-around: after you enter the vector sequence, choose "vector linearized by PCR" and "Define the position of the insert site within the vector". Enter the coordinates of the 3´ ends generated by the RE digestion. The tool will produce primers which you can ignore. While this is not ideal, it will allow you to use the tool now to generate primers for your assembly.

Q: Are you able to confirm that DNA assembly has occurred by agarose gel? In other words, is the yield high enough to visualize with ethidium bromide?

A: This really depends on the amount of starting material used and the number of fragments being assembled, as the efficiency of the reaction decreases somewhat with more fragments. Also, assembly efficiency is not 100%, so some of these gels can be difficult to read, especially in multi-fragment assemblies. We recommend checking reaction assembly by PCR directly from the assembly reaction.

Q: What keeps the exonuclease from digesting the fragments completely?

A: The size of the fragment. At the concentration provided, the enzyme can chew approximately 100 nucleotides in one hour. That is why with other mixes you cannot assemble fragments that are smaller than approx. 100 base pairs. This is not an issue with NEBuilder HiFi Master Mix, as its composition allows for the use of an oligo to insert small DNA sequences in a construct. This is illustrated in this short video.

Q: Do researchers typically perform PCR for both target and insert by utilizing a PCR machine with a gradient (because each reaction has a different annealing temperature)?

A: Yes, it is not unusual for researchers to generate their DNA fragments for assembly with PCR. If the gradient settings in your PCR machine allow you to set-up the different reactions at the recommended Tms, then this is one approach. People with thermocyclers with more limited functions often end up using two machines.

Q: Let's say we generate our linear plasmid with a restriction enzyme for use in NEBuilder. Since the process uses a 5´ exonuclease, is it important to use a RE that gives a 5´ overhang? With a 3´ overhang, doesn't one need to be more careful in what the overlapping region will be?

A: No, the overhang doesn't make a difference with NEBuilder because the polymerase in the mix will get rid of any mismatch sequence up to around 10 base pairs. In other words, you have the flexibility of designing your overlaps away from the restriction enzyme recognition site and not have to worry about that sequence during the assembly. This video illustrates this point well.

Q: What about different fragments to be assembled having the same sequence in the internal region (e.g., assembly of two expression cassettes having the same promoter)?

A: The T5 Exonuclease in the master mix can remove up to 100 nucleotides during the 1 hour incubation at 50°C. If repeated sequences, or the same sequence is >100 bp from the end of the fragment, it will not disrupt the assembly. Repeats in the overlap region that can form secondary structure in the single stranded 3´ overhang can cause problems.

Q: Can you generate a closed plasmid as the product of NEBuilder?

A: The NEBuilder HiFi DNA Assembly Master Mix contains *Taq* DNA Ligase, which will seal the nicks in the assembly, resulting in a fully ligated circular plasmid. Unlike plasmids generated by other products, this plasmid can be used as a template for PCR because it is fully sealed. It is also possible to make linear assembly products if desired; these will also be fully ligated and can also be used as PCR templates.

Q: Can you give us an estimate of the efficiency of fusing two linear ~400 bp fragments? The desired product is the 800 bp product, preferably avoiding a secondary PCR to amplify the product.

A: The NEBuilder HiFi DNA Assembly Master Mix is capable of forming linear assemblies, however, the action of the T5 Exonuclease on the linear molecule will produce recessed 5´ ends. For that reason, we recommend amplifying the assembled product. The assembly efficiency is not high, but with amplification you can produce a large amount of product.

continued on page 2

Q: Has NEBuilder HiFi been used in CHO expression systems?

A: This is an *in vitro* system used for assembling fragments, which can be then used to transfect cells. The nature of the DNA sequence is not a limitation, thus plasmid constructs containing any type of expression system can be used as a substrate for assembly.

Q: How much is the assembly affected by the design of homologous regions? Do the length and GC content of the homologous regions play a role?

A: The thing to keep in mind regarding this topic is that any stretches of Gs and Cs that may cause the formation of secondary structures at 50°C will have an effect in the efficiency of the assembly.

Q: I have a vector that includes loxP-neo-loxP, but I want to change each loxP with lox2272. Is it possible to assemble it with 2x single stranded lox2272 oligo?

A: The efficiency of assembly using two single stranded oligos at once is low. We recommend doing this in 2 rounds.

Q: I would like to know what happens if there are any mismatches in the homologous region?

A: During the assembly process the single stranded overlap regions anneal and the 3´ ends are extended by the polymerase. The polymerase does not pass through the overlap region so any mismatches will be preserved. The resulting assembled plasmid with mismatches will replicate in *E. coli* and 2 sequence variants will result. You will obtain both plasmids from each colony.

Q: If we have to clone fragments from multiple plasmids, can we do the restriction digestion simultaneously? If not, do we need to gel extract the fragments?

A: NEBuilder HiFi DNA Assembly Master Mix can be used to assemble inserts that are produced by restriction enzyme digestion of plasmids. But, the inserts must have overlaps to enable assembly with each other.

The master mix cannot assemble fragments using only the compatible cohesive ends. The plasmids can be digested together, although it is better to digest them separately so that you can check the digestion on a gel.

It is not necessary to gel extract the inserts, but column purification to remove enzyme and buffer components will increase the assembly efficiency. Mix the digested plasmids with the new vector and master mix and incubate at 50°C for 1 hour. Transform 2 µl into high efficiency competent cells.

Q: Is it possible with NEBuilder HiFi to fuse different fragments and then amplify the whole sequence by PCR?

A: Yes, that is indeed an option with this assembly method. Other assembly systems/products do not use a ligation step, which means that the assembled fragments are nicked and cannot be used in PCR.

NEBuilder-assembled products are fully ligated and serve as a good template for PCR. We recommend using 1 µl of the assembly mix as the template for PCR.

Q: Is NEB HiFi DNA assembly similar to In-Fusion® cloning?

A: In-Fusion cloning doesn't utilize a ligase to seal the nicks in the assembly and for that reason, transformation is necessary so that *E.coli* can seal the junctions. The In-Fusion assembly products cannot be used directly in PCR.

Also, the In-Fusion system is somewhat efficient for 2 fragment assemblies, but efficiency diminishes significantly with multi-fragment assemblies. There is quite a bit of comparative data on our website.

Q: Is there any evidence of better transformation efficiency using a covalently closed plasmid vs nicked?

A: Nicked and non-nicked plasmids are still structurally circular and as far as we know there is no evidence that indicates a difference in the transformation efficiency. Many cloned constructs are nicked also, since many people will dephosphorylate the vector prior to the ligation to avoid circularization and decrease background. With our many years of experience, we have not recommended a kinase step and second ligation step to obtain complete ligation of both strands in the ligation junction to subsequently obtain better transformation efficiencies.

Q: Where can I learn about considerations for performing a ssDNA oligo bridge?

A: This Application Note describes the protocol for assembling a single stranded oligo with a linearized plasmid. The application is also illustrated in this video.

Q: We have a plasmid containing two copies of a gene, and we need to introduce a point mutation into BOTH copies of the gene simultaneously. What are the key points for us to consider when using NEBuilder HiFi for this application?

A: It is difficult to mutate both copies in a single reaction because the sequences of the two genes are the same. This Application Note shows how to design primers for introducing multiple mutations simultaneously. The point mutation must be roughly in the middle of the overlap region and the primer.

However, because the two copies of the gene have the same sequence, the overlap regions will be the same. This will interfere with the order of fragment assembly since that depends on each fragment having an overlap sequence shared only by the adjacent fragment in the assembly. It might be possible to design primers that work if the mutation is close to the end of the gene since the adjacent sequence from the plasmid or the sequence between the genes is probably different.

continued on page 3

Q: What are the base pair composition limitations of the insert? Is there a known upper limit to AT content?

A: There is no limit in the AT content of the insert itself. However, it is important to keep in mind that the AT content of the overlaps needs to allow for annealing at 50°C, which is the temperature that the assembly is incubated at. As long as that sequence's Tm is above that, you are fine.

Q: What is limitation of fragment number on assembly?

A: The assembly mix formulation and the protocol recommendations that we have available with the literature of this product are optimized for assembly of 2–6 fragments. The assembly efficiency decreases every time that a fragment is added; thus a 2 fragment assembly is more efficient that a 4 fragment assembly. Assemblies with more than 6 fragments will result in lower efficiencies of assembly but have been achieved.

Q: What is the major difference between NEBuilder HiFi and Gibson Assembly® cloning?

A: NEBuilder HiFi is an assembly product that contains the three enzymatic activities used for cloning with NEB's Gibson Assembly Master Mix: a 5´ to 3´ exo, a polymerase, and a nick-sealing ligase. NEBuilder HiFi contains a polymerase activity that can handle 3' mismatch flaps, as shown in the following video and which provides some flexibility in terms of where the overlap is designed in relationship to a restriction digest site. In addition, NEBuilder HiFi is able to utilize small ssDNA oligos to assemble small sequences, as illustrated in this video.

Q: What is the reason behind 1:3 dilution of NEBuilder product before transformation?

A: This recommendation is to be used if you are using chemically competent cells from vendors other than NEB. In our testing, we and others have found that some competent cells from other vendors are susceptible to the mix components and this can cause an inhibition of the transformation step. NEB cells do not exhibit this issue and the assembly reaction can be transformed directly without dilution.

Q: What is your experience if the insert fragments are larger than 8 kb using the NEBuilder HiFi DNA assembly method?

A: NEBuilder HiFi DNA Assembly Master Mix can assemble large inserts and vectors. Large DNA fragments can be difficult to amplify. NEB recommends using a high fidelity polymerase like one of our Q5® High-Fidelity DNA Polymerase Products. The insert and/or vector can be divided into 2 smaller fragments if that makes it easier to amplify. If the vector is also large, the resulting plasmid can be difficult to transform into *E. coli*, so you may want to use electrocompetent cells for the highest transformation efficiency.

Q: What methods do you recommend for cloning highly repetitive GC rich sequences?

A: NEBuilder HiFi DNA assembly works well for most assemblies. Highly repetitive sequences are difficult because the single stranded overlap regions can form secondary structure and anneal out of register, producing sequence changes at the assembly junctions. We have several products that are appropriate for cloning highly repetitive sequences: NEB Golden Gate Assembly Mix and USER™ Enzyme. Both rely on shorter defined overhangs that will not form secondary structure and anneal correctly despite high GC content. We also recommend NEB Stable Competent *E. coli* (High Efficiency) for cloning repetitive sequences.

Q: What is the efficiency of the assembly?

A: The efficiency is dependent on a number of different variables, including the number of fragments, size of the overlap and the amount of fragments added.

Q: Why would you want to bridge dsDNA with a ssOligo rather than a dsOligo?

A: The simplest answer is because you don't need to use a dsOligo. In other words, you save yourself from having to order two oligos and from having to anneal them prior to the assembly. If you happened to have a dsOligo with the correct overlaps already in the freezer ready to go, you can certainly use that instead of the ssOligo in the application.

Q: Is treatment of the linearized vector with a phosphatase required to reduce background vector ligation in the assembly process?

A: A phosphatase treatment is not necessary. The ligase present in the mix is a nick sealing enzyme. It cannot ligate double stranded breaks, which means that it cannot circularize a fragment and cause background. Any background present in this workflow is due exclusively to an incomplete digestion.

New England Biolabs, Inc., 240 County Road, Ipswich, MA 01938-2723 Telephone: (978) 927-5054 Toll Free: (USA Orders) 1-800-632-5227 (USA Tech) 1-800-632-7799 Fax: (978) 921-1350 e-mail: info@neb.com