Improved methods for site-directed mutagenesis using NEBuilder® HiFi DNA Assembly Master Mix

Introduction

Site-directed mutagenesis (SDM) is a commonly-used technique for introducing mutations into a gene of interest. When multiple site-directed mutagenesis is needed, existing techniques, such as whole plasmid SDM, are a time consuming option that can be prone to off-target mutation incorporation. This can be a serious impediment to the planning and execution of SDM experiments.

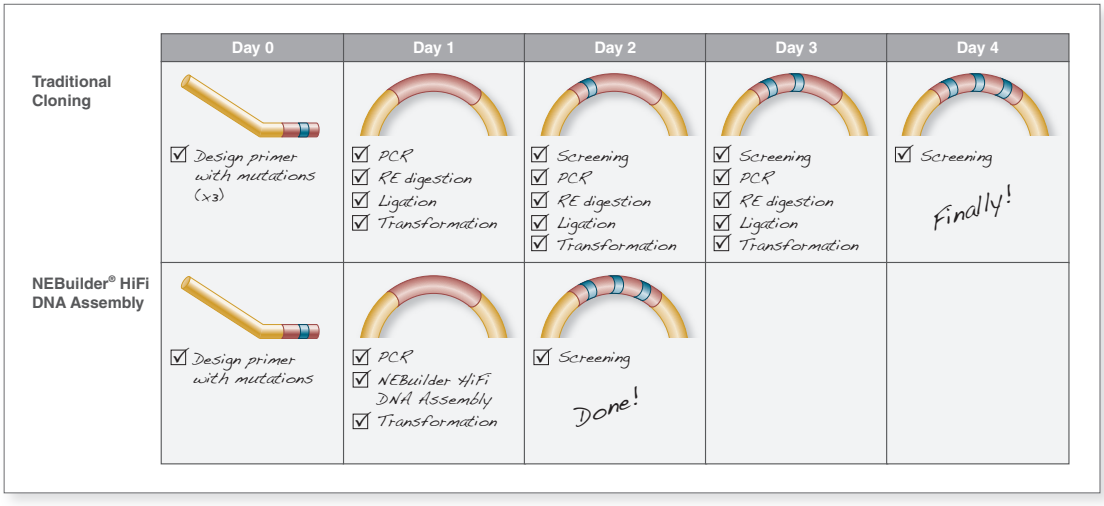
To overcome this issue, NEB® has developed a protocol using NEBuilder HiFi DNA Assembly Master Mix to simplify the construction of single/multiple site-directed mutagenesis. The technique, which involves the design of complimentary flanking primers to align fragments, can be readily adapted for SDM applications. In addition, it is unnecessary to use phosphorylated primers for assembly, reducing both cost and time. In one step, two or more PCR products with overlapping ends can be assembled into a vector. An exonuclease creates single-stranded 3′ overhangs that promote annealing of complementary fragments at the overlap region. A polymerase then fills in the gaps which are sealed by the DNA ligase. By introducing multiple complementary mutations in the primers at the overlap region, the NEBuilder HiFi DNA Assembly Master Mix forms a single, covalently bonded DNA molecule, containing the desired mutations, that is able to be directly transformed into competent cells and screened or sequenced.

Here we describe the use of the NEBuilder HiFi DNA Assembly Master Mix to generate multiple site-directed mutagenesis at the same time.

Materials

- NEBuilder HiFi DNA Assembly Master Mix
- Q5® Hot Start High-Fidelity 2X Master Mix
- EcoRI-HF®
- CutSmart® buffer (10X)
- Deoxynucleotide Solution Mix
- Overlapping primers
- *lacZ* in pET21a
- DpnI
- NEB 5-alpha Competent *E. coli*
- LB-Amp plates

Figure 1. Streamlined workflow with NEBuilder HiFi DNA Assembly



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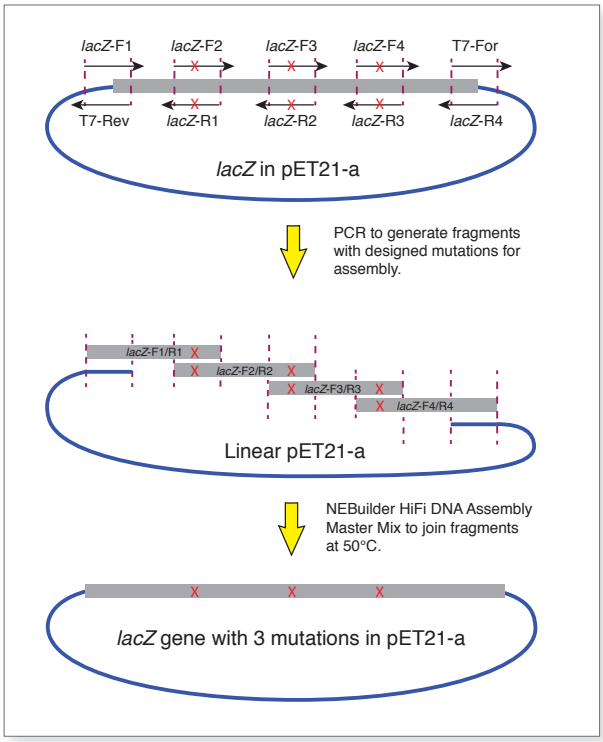
Protocol

Experimental Design

In this experiment, multiple primers were designed to incorporate 3 mutations within the gene (Table 1). The resulting amplicons contained 18-20 bp overlaps and the desired mutations (Figure 1). The PCR products and linearized vector are treated with the NEBuilder HiFi DNA Assembly Master Mix, and the resulting transformants are screened for mutations by restriction enzyme digestion.

Figure 2. Site-directed mutagenesis of *lacZ* using NEBuilder HiFi DNA Assembly.



In this example, multiple mutations are introduced into the lacZ gene through overlapping primers followed by PCR. NEBuilder HiFi DNA Assembly is then used to join the fragment with linearized vector.

Table 1. Overlapping primers used for SDM of *lacZ**

PRIMER	SEQUENCE 5´→ 3´
lacZ-F1	TTTAAGAAGGAGATATACATATGACCATGATTACGGATTC*
lacZ-R1	CACATCTGGAATTCAGCCTCCAGTACAGC**
lacZ-F2	AGGCTGAATTCCAGATGTGCGGCGAGTT
lacZ-R2	GGCCTGATGAATTCCCCAGCGACCAGAT
lacZ-F3	CTGGGGAATTCATCGCCCACGGCGC
lacZ-R3	ACACTGAGGAATTCCGCCAGACGCCA
lacZ-F4	TGGCGGAATTCCTCAGTGTGACGCTCCC
lacZ-R4	TTTGTTAGCAGCCGGATCTCATTTTTGACACCAGACCAACT
T7-For	TGAGATCCGGCTGCTAACAAAG
T7-Rev	ATGTATATCTCCTTCTTAAAGTTAAACAAAAT

Red indicates changed nucleotides.

Fragment Preparation:

1. The following reaction conditions were used to amplify fragments with designed mutations using the following primer pairs: *lacZ*-F1/R1, *lacZ*-F2/R2, *lacZ*-F3/R3, *lacZ*-F4/R4 and T7-For/T7-Rev.

COMPONENT	VOLUME (µl)
Forward primer (10 µM)	2.5
Reverse primer (10 µM)	2.5
lacZ in pET21a (5 ng/µl)	1.0
ddH_2O	19.0
Q5 Hot Start High-Fidelity 2X Master Mix	25.0
Total Volume	50.0

STEP	TEMP	TIME
Initial denaturation	98°C	1 minute
30 Cycles	98°C	10 seconds
	55°C	15 seconds
	72°C	40 sec. or 3 min
Final extension	72°C	5 minutes
Hold	4°C	∞

2. Following PCR, 1 µl of DpnI was added to each tube and incubated at 37°C for an additional 30 minutes.
3. After DpnI treatment, all products were cleaned up using Qiagen QIAquick™ PCR purification columns.

Fragment Assembly:

1. Concentration of the fragments was determined by Nanodrop™ instrument or estimated by agarose gel electrophoresis.
2. The 2X NEBuilder HiFi DNA Assembly Master Mix was thawed at room temperature.
3. The NEBuilder HiFi DNA Assembly reaction was set up as follows:

COMPONENT	AMOUNT
Vector	0.05 pmols
PCR products (for each fragment)	0.05 pmols
2X NEBuilder HiFi DNA Assembly Master Mix	10 µl
H_2O	10-x µl
Total volume	20 µl

x = total volume of fragments (including vector)

4. The reaction was incubated at 50°C in a thermocycler for 1 hour.
5. 2 µl of the reaction was transformed into 50 µl of NEB 5-alpha Competent *E. coli* (High Efficiency), plated on LB-Amp plates and incubated overnight at 37°C.

Results:

Transformation resulted in several thousand colonies. Ten colonies were screened by EcoRI-HF restriction enzyme digestion, 8 of which contained the desired mutations (data not shown).

Summary

These results demonstrate the versatility of the NEBuilder HiFi DNA Assembly Master Mix in multiple site-directed mutagenesis. In this example, three mutations were introduced into the *LacZ* gene simultaneously. Four overlapping PCR amplicons were assembled with a linearized vector in one step. Resulting colonies were screened by sequencing, 80% of which contained the desired mutations. This represents a substantial improvement over earlier methods of multi-site mutagenesis. Whereas previously, one may have had to create mutations sequentially, leading to a significant increase in the length of the experiment, SDM using NEBuilder HiFi DNA Assembly Master Mix can be done in one step and in much less time.

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

Ordering Information

PRODUCT	NEB #	SIZE
NEBuilder HiFi DNA Assembly Master Mix	E2621S/L/X	10/50/250 reactions
COMPANION PRODUCTS		
NEBuilder HiFi DNA Assembly Cloning Kit	E5520S	10 reactions
NEB 5-alpha Competent *E. coli* (High Efficiency)	C2987I/H	6 x 0.2 ml/ 20 x 0.05 ml
Q5 Hot Start High-Fidelity 2X Master Mix	M0494S/L	100/500 rxns
DpnI	R0176S/L	1,000/5,000 units
EcoRI-HF	R3101S/L	10,000/50,000 units

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