Improved Method for Assembly of Linear Yeast Expression Cassettes using NEBuilder® HiFi DNA Assembly Master Mix

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Introduction

Heterologous protein production in yeast expression systems (i.e., *Kluyveromyces lactis* and *Pichia pastoris*) normally involves insertion of a linear expression cassette into a target locus in the host genome (1-3). Typically, an expression cassette is assembled in *E. coli* by first cloning a gene of interest into a circular expression vector (Figure 1A). The expression cassette comprises DNA encoding a strong yeast promoter upstream of a heterologous gene of interest, a downstream transcription terminator sequence and a selectable marker gene. The entire cassette is flanked by locus-specific targeting sequences on either end (Figure 1B). The assembled vector is then amplified by propagation of the host *E. coli* cells, isolated by standard DNA preparation techniques, and subjected to restriction digestion to create a linear expression cassette. The linear fragment is then introduced into yeast whereby it integrates into a target locus on the host chromosome (Figure 1C).

While this approach for expression strain construction has been accepted methodology for over two decades, it does have some limitations. For example, expression vector assembly using traditional cloning techniques is a multi-day process. Additionally, one is limited to the use of restriction sites that are present in the expression vector and the use of the expression machinery (i.e., promoter, terminator, locus targeting sequence, etc.) that is built into the vector. Finally, for cloning of a gene whose product is highly toxic to *E. coli*, serendipitous protein expression during vector assembly in bacteria can yield clones having deleterious mutations (1,4). The use of *in vitro* DNA assembly methods to construct linear expression cassettes suitable for direct introduction into yeast circumvents each of these limitations while shaving days off of expression strain construction time. In the presented method, we highlight the use of NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621) to rapidly assemble expression cassettes for use with NEB's *K. lactis* Protein Expression Kit (NEB #E1000).

Materials

NEBuilder HiFi DNA Assembly Master Mix

Q5® Hot Start High-Fidelity 2X Master Mix

Overlapping Primers

NEBuilder Assembly Tool

K. lactis Protein Expression Kit

pKLAC2

K. lactis GG799 Competent Cells

Yeast Carbon Base Medium

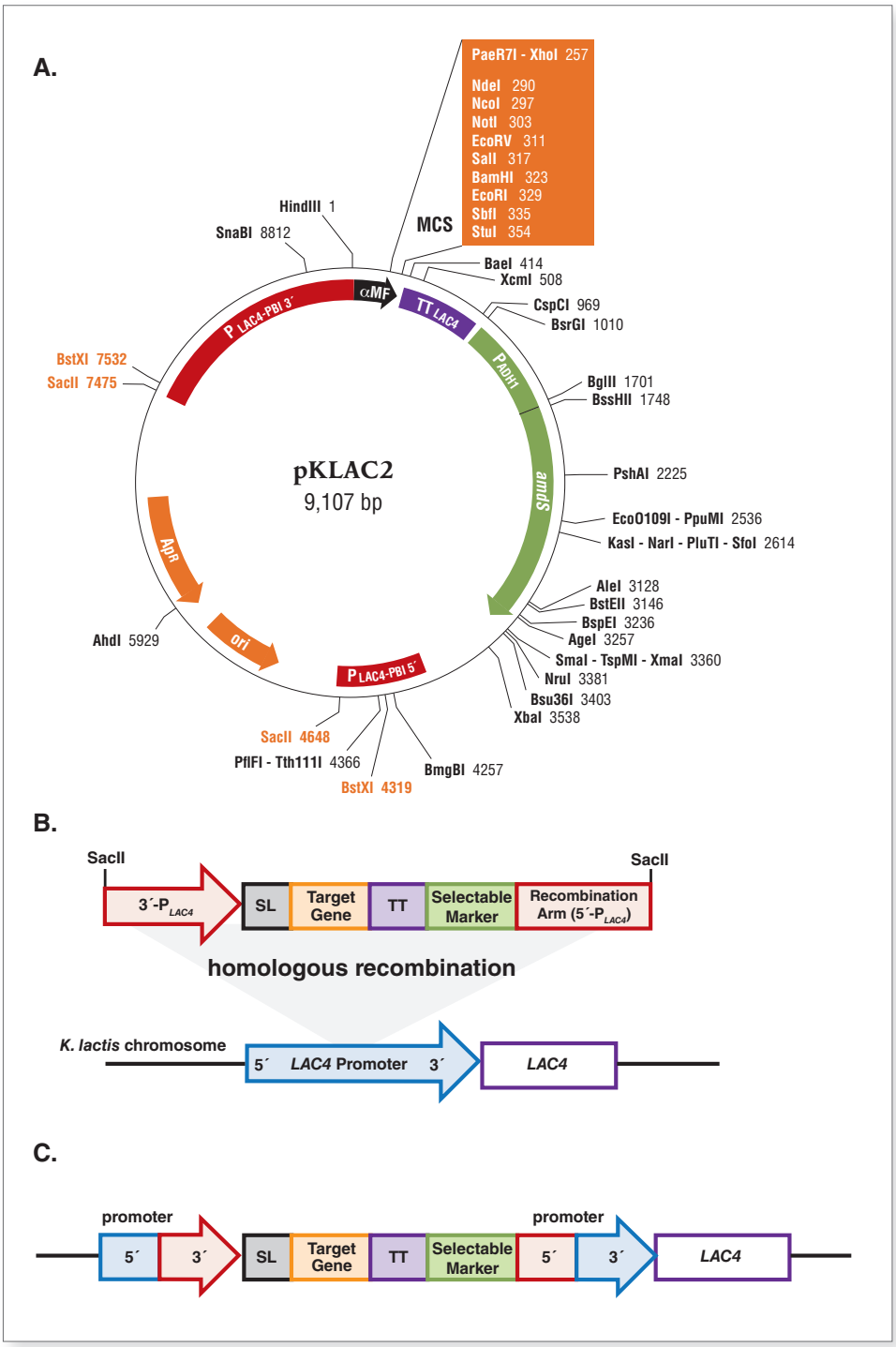
Biolux® *Gaussia* Luciferase Assay Kit

References:

1. Colussi, P.A. and Taron, C.H (2005) *Kluyveromyces lactis LAC4* promoter variants that lack function in bacteria but retain full function in *K. lactis*. *Appl. Environ. Microbiol.* 71:7092–7098.
2. van Ooyen, A.J.J., Dekker, P., Huang, M., Olsthoorn, M.M.A., Jacobs, D.I., Colussi P.A. and Taron, C.H. (2006) Heterologous protein production in the yeast *Kluyveromyces lactis*. *FEMS Yeast Res.* 6:381–392.
3. Read, J.D., Colussi, P.A., Ganatra, M.B. and Taron, C.H. (2007) Acetamide selection of *Kluyveromyces lactis* cells transformed with an integrative vector leads to high frequency formation of multicopy strains. *Appl. Environ. Microbiol.* 73:5088–5096.
4. Gibbs, M., Reeves, R.A., Sunna, A., and Bergquist, P.L. (2004) A yeast intron as a translational terminator in a plasmid shuttle vector. *FEMS Yeast Res.* 4:573–577.

FIGURE 1: **Traditional yeast expression strain construction.**

(A) The pKLAC2 integrative expression vector. A target gene is cloned into the multiple cloning site (optionally, in-frame with the mating factor alpha secretion leader for extracellular expression) using traditional techniques. Transcription is initiated and terminated by the LAC4 promoter (PLAC4-PB1) and LAC4 transcription terminator (TTLAC4) sequences, respectively. The *Saccharomyces cerevisiae* ADH1 promoter (PADH1) drives expression of a fungal acetamidase gene (amdS) for the selection of transformants by growth on acetamide-containing medium. *E. coli* vector sequence has been inserted into a unique SacII site in PLAC4 to allow for propagation in bacteria. (B) Targeted integration of a linear expression cassette. The vector is linearized by digestion with SacII or BstXI to permit subsequent insertion into the LAC4 promoter locus in the *K. lactis* genome. In the illustrated example, the fragment becomes inserted into the LAC4 promoter locus by recombination, mediated by homologous targeting sequences at either end of the fragment. The resulting chromosomal arrangement is shown in (C). Abbreviations: 3´-PLAC4, left targeting sequence; 5´-PLAC4, right targeting sequence; SL, secretion leader; TTLAC4 transcription terminator.



Protocol

Experimental Design – Liner expression cassette assembly

In this experiment, multiple pairs of primers were designed using the NEBuilder Assembly Tool (nebuilder.neb.com) to permit amplification of individual functional domains of an expression cassette (Table 1). Each resulting amplicon contained 22 bp overlaps with adjacent domains (Figure 2A). Purified PCR products were combined and treated with NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621) and the fully assembled expression cassette was amplified using primers P1 and P12 (Figure 2B) to obtain sufficient quantities for introduction into *K. lactis* cells.

Fragment Preparation

1. The following reaction conditions were used to amplify individual functional domains of the expression cassette (schematic in Figure 2A, primers in Table 1).
2. After thermocycling, each amplicon was purified by gel extraction.

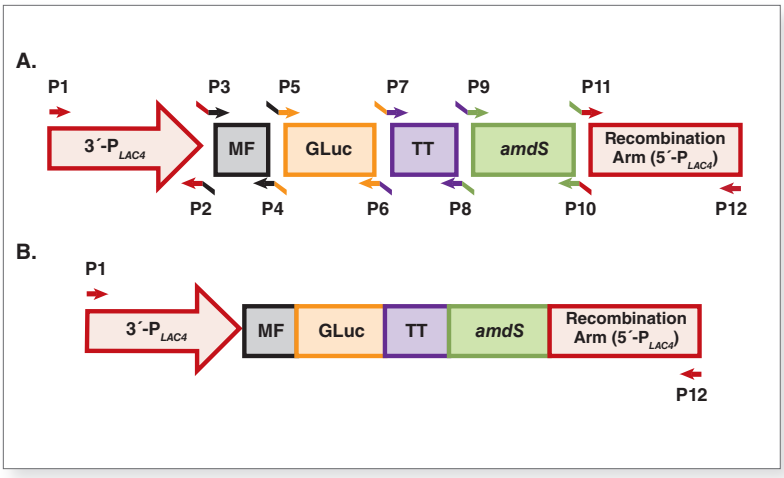
TABLE 1: **Overlapping primers used for NEBuilder assembly of a linear *K. lactis* expression cassette.**

PRIMER	SEQUENCE 5´→ 3´
P1	GATCGACTCATAAAATAGTAACC
P2	GAGAATTTCATTTTTTTCAAGCTTCTCGATG
P3	GCTTGAAAAAAATGAAATTCTCTACTATATTAGCCG
P4	TCGGTGGGCTTTCTTTTCTCGAGATCATCC
P5	TCGAGAAAAGAAAGCCCACCGAGAACAACG
P6	AAGGGGCCTGTTTAGTCACCACCGGCCCC
P7	GTGGTGACTAAACAGGCCCCTTTTCCTTTG
P8	CACCCGGAAACAGCTTGCAAATTAAAGCCTTC
P9	ATTTGCAAGCTGTTTCCGGGTGTACAATATG
P10	ATCTGTTCCTTCTATGGAGTCACCACATTTC
P11	TGACTCCATAGAAGGAACAGATAGATAAAATTCCG
P12	CCGCGGAAA0TTTAGGAATTTTAAAC

*Colors indicate DNA regions corresponding to the different functional domains shown in Figure 2A.

FIGURE 2: ***In vitro* assembly of a linear expression cassette.**

(A) Primer pairs P1 through P12 were used in PCR to amplify individual functional domains of the expression cassette.
(B) The domains were then assembled using the NEBuilder HiFi DNA Assembly Master Mix and the assembled linear expression cassette was amplified by PCR using primers P1 and P12. In this experiment, the CYC1 transcription terminator (TT) was used.



Expression Cassette Assembly

1. The concentration of individual functional domain amplicons was determined by a Nanodrop™ instrument or estimated by agarose gel electrophoresis.

COMPONENT	VOLUME (µl)
Forward primer (40 µM)	1.25
Reverse primer (40 µM)	1.25
DNA template (5 ng/µl)	1
ddH_2O	46.5
Q5 Hot Start High-Fidelity 2X Master Mix	50
Total Volume	100

STEP	TEMP	TIME
Initial denaturation	98°C	30 seconds
30 Cycles	98°C	10 seconds
	58°C	30 seconds
	72°C	30 seconds or 1 minute
Final extension	72°C	7 minutes
Hold	4°C	∞

2. NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621) was thawed at room temperature.

3. The DNA assembly reaction was set up us follows:

COMPONENT	AMOUNT
PCR Amplicons	0.03 pmol (each)
2X NEBuilder HiFi DNA Assembly Master Mix	10 µl
ddH_2O	x µl
Total Volume	20 µl

4. The reaction was incubated at 50°C for 1 hour.

5. A 2 µl aliquot of the reaction was used as template in a fresh PCR reaction using primers P1 and P12 to amplify the assembled cassette. (Note: occasionally, more than one 100 µl reaction is needed to generate enough pooled assembled product for introduction into yeast).

6. Following thermocycling, the amplicon was purified by gel extraction.

COMPONENT	VOLUME (µl)
Forward primer (40 µM)	1.25
Reverse primer (40 µM)	1.25
DNA Template (fragment assembly mix)	2
ddH_2O	45.5
Q5 High-Fidelity 2X Master Mix	50
Total Volume	100

STEP	TEMP	TIME
Initial denaturation	98°C	30 seconds
30 Cycles	98°C	10 seconds
	58°C	30 seconds
	72°C	3 minutes
Final extension	72°C	7 minutes
Hold	4°C	∞

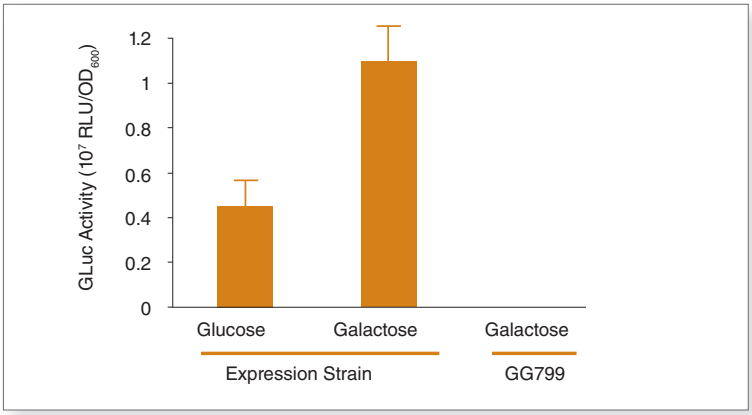
7. An aliquot containing 0.1–1 µg of the assembled linear expression cassette was used to transform *K. lactis* GG799 Competent Cells (NEB #C1001). The transformation reaction was plated on Yeast Carbon Base Medium (NEB #B9017) containing 5 mM acetamide and incubated for 3-4 days at 30°C until colonies formed. Targeted integration of the linear expression cassette into the *K. lactis* genome was confirmed by colony PCR using standard techniques described in the *K. lactis* Protein Expression Kit Manual (NEB #E1000).

Results

A transformation efficiency of about 8 x 10^3 colonies/µg DNA was obtained with the *in vitro* assembled expression construct, a similar efficiency to that observed when using a cloned expression cassette liberated from a pKLAC2 expression construct. *K. lactis* cells harboring at least one integrated copy of the *in vitro* assembled expression cassette successfully secreted *Gaussia princeps* luciferase (GLuc) into the growth medium (Figure 3).

FIGURE 3: **Expression of *Gaussia princeps* luciferase (GLuc) in *K. lactis* GG799 cells.**

The NEBuilder-assembled linear expression cassette (shown in Figure 2B) was used to transform *K. lactis* GG799 cells. A representative transformant was cultured in triplicate in rich medium (yeast extract/peptone) supplemented with glucose or galactose as a carbon source. Untransformed GG799 cells were grown in medium containing galactose as a negative control. GLuc enzyme activity secreted into the growth medium was measured using the BioLux *Gaussia* Luciferase Assay Kit (NEB #E3300). GLuc expression from the LAC4 promoter is repressed in the presence of glucose, but is de-repressed in galactose, as expected (1-3).



Conclusion

In this application note, the use of NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621) to rapidly assemble a linear expression fragment for introduction into *K. lactis* was demonstrated. The method permits fast preparation of linear expression cassettes in 1-2 days whereas the classical *E. coli* cloning approach takes at least 4-5 days. The *in vitro* assembly method is also suitable for the cloning of toxic genes because it eliminates the potential for accumulation of deleterious mutations during expression vector construction steps in bacteria. Moreover, NEBuilder HiFi DNA assembly permits more flexibility in the creation of constructs having different promoters, signal sequences, reporter genes and integration sites compared to using pKLAC2 in the *K. lactis* Protein Expression Kit (NEB #E1000). The general methodology outlined in this application note can also be applied more widely to assemble linear DNAs containing expression machinery for introduction into other yeasts (e.g. *Pichia pastoris*), fungi, bacteria, plant cells or animal cells.

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