Integrating Synthetic Gene Assembly and Site-Specific Recombination Cloning

Bianca J Lam
+ 1 7602688518
bianca.lam@lifetech.com

Frederico Katzen
+ 1 7604767145
federico.katzen@lifetech.com

Kevin Clancy
+ 1 7602688356
kevin.clancy@lifetech.com

ABSTRACT
We developed an *in vitro* workflow streamlined to assemble full length genes from synthetic fragments directly into expression vectors for testing in a wide range of organisms. The genes of interest are first divided into small fragments for synthesis and are constructed with homology to other fragments or expression vector. The fragments are assembled into larger subfragments by high-fidelity PCR. For genes shorter than 12 kb, the gene subfragments and an expression vector are added to an enzymatic mix that assembles the subfragments and vector in the correct order and orientation. Finally, the enzymatic reaction is transformed into *E. coli* for plasmid propagation and screening resulting in seamless full length genes unmodified by extra or missing sequences caused by traditional cloning techniques. For genes larger than 12 kb, gene subfragments were first assembled into pUC19 and then through a second round of homologous recombination cloning, assembled into full length genes into the final expression vector. Assembled genes can also be site-specifically recombined to make multiple expression plasmids containing different elements and/or tags thereby circumventing the need to sequence re-verify gene. Thus, this technology allows for simultaneous testing of the genes of interest in bacteria, yeast, algae, plants, insect, and/or mammalian cells. The described workflow is currently being adapted for an automated high-throughput platform for the concurrent construction of multiple full-length genes. These technologies combined with computer-aided design of strategy, screening, automation, and LIMS will greatly advance gene editing, protein engineering, synthetic pathway engineering, and host engineering efforts.

Categories and Subject Descriptors
J.3. [**Life and Medical Sciences**]: *biology and genetics*

General Terms
Design, Experimentation

Keywords
Synthetic biology, synthetic genes, gene assembly, cloning, metabolic engineering

1. INTRODUCTION
Current methods to engineer biological systems and processes include gene and/or genome assembly, which requires advance cloning technologies. Homologous recombination cloning utilizes terminal end-homology between DNA fragments resulting in the directional and seamless insertion of multiple DNA fragments into a cloning vector. Using homologous recombination cloning, ~444 synthetic gene fragments were assembled into forty-four 7-27 kb full length genes with high cloning efficiency. Assembled genes were also exchanged into other vectors without the need for re-sequencing using site-specific recombination cloning. With computer-aided design of cloning strategies and screening of assembled genes, automation, and a LIMS for sample tracking, we aim to increase the efficiency and robustness of this synthetic gene assembly workflow.

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2. RESULTS

2.1 Gene Assembly Strategy and Design
Full length genes ranging from 7-27 kb were divided into ~1 kb fragments. Fragments contained homology to vector and/or adjacent fragments. Gene fragments were received sequence confirmed in a pMX cloning vector from GENEART®. Forward and reverse primers used for PCR amplification of the fragments hybridized to regions of overlap. The first and last primer contained homology to pcDNA-Dest40 (Invitrogen) or pUC19. Additional primers used for full length sequencing were located in the middle of 1 kb fragments.

2.2 First PCR
The 1 kb fragments were first PCR amplified and treated with Dpn I to digest supercoiled plasmid template.

2.3 Assembly PCR
PCR products from two or three consecutive 1 kb gene fragments were combined for assembly PCR reactions to create larger gene subfragments.

2.4 Seamless DNA Fragment Assembly
For genes ≤12 kb, the subfragments, linearized pcDNA-Dest40 vector (Invitrogen), and GENEART® Seamless Cloning enzyme were incubated at room temperature for one hour. Reactions were transformed into Top 10 competent cells (Invitrogen) for plasmid propagation and screening. For genes >12 kb, gene subfragments were first assembled into pUC19 as 4-6 kb fragments. The subfragments were then released from pUC19 by either PCR amplification or restriction digest for a second round of homologous recombination cloning into the desired plasmid.

2.5 Screening for Full Length Genes
Full length genes assembled into vector were screened by restriction digestion. Restriction endonucleases for screening were chosen based on the expected banding pattern of the cloned sequence. Four clones that passed restriction digestion screening were selected for full length sequencing. On an average, ≥25% of clones with the correct banding pattern also matched the predicted sequence 100%.

2.6 Integration into Site-Specific Recombination Cloning

Gene constructs with 100% sequence match to the predicted sequence underwent site-specific recombination cloning with pDONR™221 (Invitrogen) or equivalent to create Entry vectors. Entry vectors can be site-specifically recombined into any Destination vector to create respective expression plasmids.

3. DISCUSSION/CONCLUSIONS

The cloning technologies and workflow discussed here consist of a robust method for assembling and cloning large genes and/or DNA fragments from smaller parts. For assembling PCR-amplified genes or large gene subfragments (up to 12 kb) into vector, we observed a range between 10% and 80% of picked colonies contained all fragments in the vector. Typically, a minimum of 25% of plasmids containing all DNA fragments also had 100% sequence match. We suspect that sequence content of the genes and the ad hoc method of fragment and primer design contributed to the wide range of variation in cloning efficiency. However, we found that primer quality was the critical factor for the success of this method. The use of HPLC or PAGE purified primers increased the success of fragment assembly and genes with 100% sequencing match. Assembly of pre-cloned gene subfragments into an expression vector was similarly efficient where a range between 17% and 83% of picked colonies contained all fragments. We are in the process of implementing computer-aided design software, such as Vector NTI®, to choose optimal sites for gene fragmentation, designing PCR primers and to devise a screening strategy for assembled DNA fragments. Communication between the CAD software and LIMS will facilitate sample tracking and streamline each step within the cloning procedure.

4. ADDITIONAL AUTHORS

Kimberly Wong (kimberly.wong@lifetech.com,+ 1 7602688405), Todd Peterson (todd.peterson@lifetech.com, + 1 7604767180), and Antje Pörtner-Taliana (antje.taliana@lifetech.com, + 1 7604766842).

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