Robotic Automation of High Throughput Cloning

To clone a large number of genes from genomic DNA or cDNA libraries, and to subclone each gene into multiple expression vectors, we have sub-divided the entire process from PCR to mini-expression screening into 9 steps. All steps are robotized using a 96 well format.

The basis of our cloning methodology is a BSGC developed Ligation-Independent Cloning [LIC] system (Aslanidis and de Jong, 1990) that allows for rapid and efficient Target cloning into a series of N-terminal fusion vectors. The majority of our vectors are designed to express Targets as 6-Histidine + Tobacco Etch Virus (TEV) Protease cleavable fusions with an N-terminal 5-glycine LIC Linker that allows for efficient TEV cleavage (D.Waugh, personal communication). The current vector set as listed in Table 1 consists of five TEV-cleavable vectors and two additional vectors, one with no tag and the other with a non-cleavable 6HisTag. The C-terminal LIC Linker permits Target fusion to GFPuv, a potential indicator of soluble protein expression, when expressed in a LE392(DE3) host strain that suppresses TAG codon termination.

Our current robotic platform is based on the Biomek2000 (Beckman Coulter, Fullerton, CA) utilizing BSGC developed protocols that are adaptable to the higher throughput BiomekFX platform. Two essential innovations that has enabled an efficient robotic cloning protocol are (1) our LIC vector system with a greater than 90 percent cloning efficiency and (2) the ability to immediately assess clones by mini-expression in the BL21(DE3) expression host. By preserving the initial colony selections, rapid large-scale protein production of any promising target clones can immediately follow.

Table 1. BSGC LIC Expression Vectors

Vector Name	Fusion
pB1	None
pB2	6-Histidine
pB3	6-Histidine+TEV
pB4	6-Histidine+Maltose Binding Protein+TEV
pB5	6-Histidine+Glutathione S-Transferase+TEV
pB6	6-Histidine+Thioredoxin+TEV
pB7	6-Histidine+NusA+TEV

Stages of Automated Cloning:

1. PCR Amplification

   Five nanomoles of primers for 96 Targets are automatically designed and ordered in two 96 well plates, one for the N-terminus and the other for the C-terminus of the Target. The robotic protocol resuspends the primers and combines aliquots in the PCR reaction mixture plate together with template and other PCR reaction components.

2. PCR Product Analysis
   Following PCR amplification, the reaction products are assessed by E-Gel ® (Invitrogen, Carlsbad, CA) that are robotically loaded and run using an in-house developed protocol. Gel data is captured electronically and processed for analysis.

3. PCR Product Purification
   Amplified DNA products are purified using commercial kit components (Promega, Madison, WI) consisting of 96 well resin/filtration plates.

4. PCR Quantitation
   Aliquots of DNA are quantified by Picogreen (Molecular Probes, Inc.) fluorescence and/or by E-gel fluorescence. All products are carried through the protocol regardless of yield since even very low amounts of PCR products can yield good LIC clones.

5. Insert Preparation
   Aliquots of DNA products in a 96 well format are prepared for insertion by reaction with T4 DNA polymerase in the presence of dATP. These plates provide multiple insert stocks, typically for up to 20 LIC reactions.

6. LIC Cloning Reaction
   The prepared Target Insert is combined with Prepared LIC Vector in a room temperature reaction. Typically this reaction is combined with the transformation step described below in a single robotic protocol.

7. Host Cell Transformation
   The LIC Reaction is transformed into high-efficiency competent Expression Host BL21(DE3) and is plated using the Petristrip Method (McNutty and Dunn, 1999).

8. Colony Screening, Insertions
   Two colonies from each reaction are picked into 96 well growth plates containing 1 ml of LB medium. Plates are grown for 4 hours at 37 C and used for inoculating mini-expression plates and stab cultures. The remaining culture is grown overnight at 37 ^{o}C for preparation of plasmids.

9. Colony Screening/Mini-Expression
   Growth plates containing 250 ul of ZYP autoinduction media (Novagen, Madison, WI) are inoculated with 50 μl of colony culture and grown overnight at 37 ^{o}C for analysis by denaturing SDSPAGE. Plates for plasmids are processed using an economical BSGC developed Biomek protocol that is sufficient for plasmid preservation and screening.

<u>References</u>

Aslanidis C, de Jong PJ. (1990) Ligation-independent cloning of PCR products (LIC-PCR). Nucleic Acids Res. 20:6069-74.

McNulty JJ, Dunn JJ. (1999) High-throughput transformation and plating using petristrips. Biotechniques. 3:390-2.

Contact Person: Hisao Yokota 510-486-4332; e-mail: HAYokota@lbl.gov