GenScript LIC Kit

Technical Manual No. 0180 **Version 20050515**

I. DESCRIPTION

Genscript LIC Kit (SD0221) is designed for rapid and efficient cloning of genes into Genscript pDream2.1 vector for the expression of the genes in any one of the three major protein expression systems: bacteria, insect cells and mammalian cells. By using ligation-independent cloning (LIC) techniques, one can use the predigested vector provided in the kit for the cloning of any genes without further restriction enzyme treatment. With this technology, selection of restriction enzymes is no longer necessary, no restriction enzymes, ligase, or blunt-end polishing are needed. You just need to amplify the gene by PCR using appropriately designed primers. After treatment with T4 DNA polymerase in the presence of dATP, the PCR DNA insert is annealed to the vector in a 10-minute incubation. The annealing reaction is used directly to transform *E. coli* [1, 2], and the correct clones will be selected by mini-preps and restriction enzyme digestion.

LIC technology is outlined and shown in Figure 1 on the right.

Outline of LIC Cloning Method

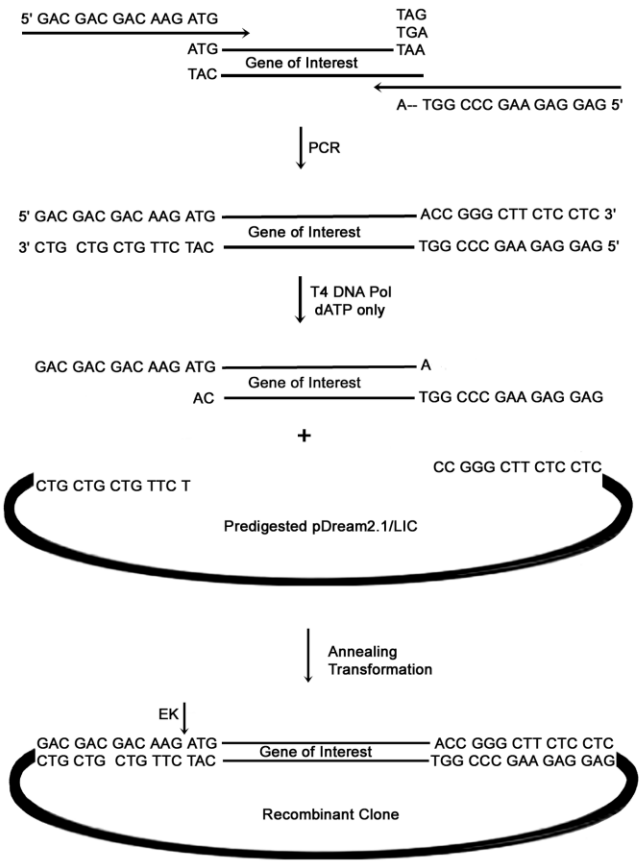


Figure 1. Outline of LIC cloning method.

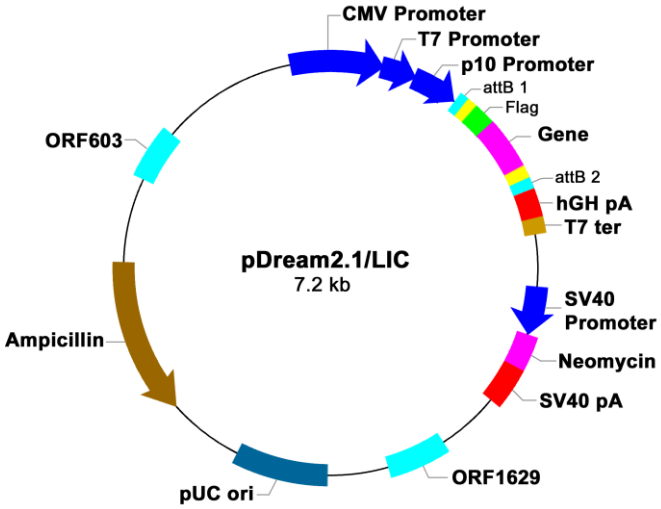
II. KIT COMPONENTS

The kit (SD0221) contains the following components:

Predigested pDream2.1/LIC	1 µg	20 µl
T4 DNA Polymerase	20 U	20 µl
5X T4 DNA Polymerase Buffer	5X	100 µl
cGFP Control Insert	5 ng/µl	10 µl
dATP	25 mM	50 µl
EDTA	25 mM	50 µl

III. VECTOR DESCRIPTION

GenScript pDream2.1/LIC vector (Figure 2) is a protein expression vector for both efficient cloning and high-level expression of any target gene. The gene of interest can be efficiently cloned into the vector using Ligation Independent Cloning (LIC) strategy, and can be expressed directly without any further cloning work in any one of the three major protein expression systems: Bacteria, Insect cells and Mammalian cells.



Predigested Vector:

Promoters and forward sequencing primer →

ACAAGTTTGTACAAAAAAGCAGGCTAAGGAGATATACC**ATGGATTACAAGGATGACGACGACAAGA** ← sticky end

attB1 **FLAG Sequence & EK Cleavage**

sticky end → CCGGGCTTCTCCTCAGCGGCCGC**CTATAGTGTCACCTAAATCGT**A**ACCCAGCTTTCTTGTACAAAGTGGT**

Reverse Sequencing Primer **attB2**

→ **hGH polyA signal and T7 terminator**

Figure 2. The circle map, the positions of key features and the cloning region of pDream vector

Sequencing Primers:

Forward primer: T7 sequencing primer, DA0009, at T7 promoter
Reverse primer: SP6 sequencing primer, DA0008.

IV. PCR PRIMER DESIGNING

The forward and reverse primers must include two special sequences at the 5'-ends of the primers for LIC cloning purpose. For optimal PCR amplification, the use of purified primers is highly recommended. The

Forward primer: 5'- GAC GAC GAC AAG **ATG ----, 5'-end coding sequence starting with ATG**
Reverse primer: 5'- GAG GAG AAG CCC GGT ----, **3'-end coding sequence including stop codon(s)**

V. LIC CLONING PROCEDURE

PCR amplification and purification

1. Amplify the gene sequence using the primers containing the sequences as described above. To reduce the likelihood of DNA mutations during PCR reaction, we recommend using DNA polymerases with low mutation rates, such as *Pfu* DNA polymerase or KOD DNA polymerase (Novagen, Cat. No. 71086-3), etc.
2. Analyze PCR results by agarose gel electrophoresis. If the PCR reaction is very clean with little background and no extraneous bands, PCR product can be purified using PCR purification kit and then used in the next step: T4 DNA polymerase treatment. **dNTPs from PCR reaction must be completely removed for successful T4 DNA polymerase treatment.** However, if the PCR template has the same antibiotic resistance marker as this vector (Amicillin), gel purification of PCR product is needed to avoid false positives.
3. If the PCR reaction produces high background with extraneous bands, or the PCR template has the same antibiotic resistance marker as this vector (Amicillin), gel purification of PCR product is necessary.
4. Elute or resuspend purified PCR product in TE buffer.

Insert preparation: T4 DNA polymerase treatment of purified PCR product

1. Set up the T4 DNA polymerase treatment reaction as follows:
   - 2 µl purified PCR product (50 ng for 500 bp insert, 100 ng for 1 kb insert, etc.)
   - 4 µl 5X T4 DNA Polymerase Buffer
   - 2 µl 25 mM dATP
   - 11 µl ddH2O
   - 1 µl T4 DNA Polymerase (1 U)

   Mix well by pipeting up and down several times, and incubate at 22 – 25 ^{o}C for 30 minutes.
2. Inactivate T4 DNA polymerase treatment to stop the reaction by incubating at 75^{o}C for 20 minutes.
3. The treated insert can be used in annealing right away or stored at - 20^{o}C for future use.

Annealing the insert and vector

Mix 1 µl predigested vector with 2 µl of T4 DNA polymerase treated insert in a 1.5 ml microcentrifuge tube and incubate at 22 ^{o}C for 5 minutes. Place the tube on ice and the mixture will be directly used for transformation.

Transformation

Commonly used competent *E. coli* cells, such as DH5α, BL21(DE3) competent cells, can be used for this purpose.

1. Thaw *E. coli* DH5α (or BL21(DE3)) competent cells on ice. Pipet 50 µl of DH5α competent cells into 1.5 ml microcentrifuge tube containing the annealing mixture. Gently mix well and incubate on ice for 30 minutes.
2. Heat shock at 42^{o}C for 45 seconds. Incubate on ice again for two minutes.

3. Add 500 ml LB. Incubate at 37°C with shaking at 250 rpm for one hour.
4. Plate 100 µl of the cells on one LB/Ampicillin (100 µg/ml) agar plates, and the remaining cells on another plate.
5. Incubate plates overnight at 37°C.
6. Pick single colonies for colony screening.

VI. EXPRESSION IN MAMMALIAN CELLS.

Generate large amount of DNA

If you need large amount of DNA for transfection, perform a MaxiPrep using Qiagen MaxiPrep kit.

General considerations before transfection

A variety of protocols such as lipofection and electroporation have been used successfully to transfect gene constructs into mammalian cells. The transfection procedures are identical to those used for DNA plasmid transfection. The choice of transfection procedures will depend on the mammalian cell line used. In general, we recommend using Lipofectamine™ 2000 or LipofectaminTM Plus from Invitrogen. The information and protocol for Lipofectamine™ 2000 can be found using this link: http://www.lifetech.com/content.cfm?pageid=93. The information and protocol for Lipofectamine™ Plus can be found using this link: http://www.invitrogen.com/content/sfs/manuals/18324.pdf.

The following are important issues to be considered before performing the transfection:

a. **Cell density:** The recommended cell density for transfection using Lipofectamine™ 2000 is 90-95%. If the cell density is less than 90%, the Lipofectamine™ 2000 may have some toxicity on the cells.
b. **Human gene construct amount:** For 12-well plates, it is recommended to use 1.6 µg as a starting point. For other plate size, the DNA amount can be adjusted proportionally.
c. **Cell Proliferation:** Maintaining healthy cell culture is critical for cell transfection. It is essential to minimize decreased cell growth associated with nonspecific transfection effects.
d. **Time:** The optimal time after transfection for analyzing gene expression has to be determined empirically by testing a range of incubation time. The time can vary from 24 to 96 hrs depending on the cells used and the human genes tested.

A protocol based on Lipofectamine™ 2000 from Invitrogen for 12-well plates

1. Purchase Lipofectamine™ 2000 reagent (Cat#11668-027) from Invitrogen
2. Plate the cells the day before transfection so that they are 90-95% confluent on the day of transfection. At the time of plating cells and diluting transfection reagents, avoid antibiotics - this helps cell growth and allows transfection without rinsing the cells. The cell density of 90-95% is very important. If the cell density is less than 90%, transfection may have toxicity on the cells.
3. Dilute the human gene construct with 100 µl of Opti-MEM (Cat# 31985062) from Invitrogen and mix gently.
4. Dilute Lipofectamine™ 2000 Reagent into 100 µl of Opti-MEM medium in a second tube; mix gently and incubate at room temperature for 5 min.
5. Combine diluted DNA (from Step 3) and diluted Lipofectamine™ 2000 Reagent (from step 4); mix and incubate at RT for 20 min.
6. Add 200 µl of DNA-Lipofectamine™ 2000 Reagent complexes to each well. Mix gently by rocking the plate back and forth.
7. Perform assays on the cells 24-48 h after the start of transfection. It is not necessary to remove the complexes or change the medium; however, growth medium may be replaced after 4-6 hours without loss of transfection activity.
8. Optimizing transfection: The suggested starting points are listed in Table 1. To obtain the highest transfection efficiency and low non-specific effects, optimize transfection conditions by varying DNA and Lipofectamine™ 2000 concentrations, and cell density. Make sure that cells are greater than 90% confluent and vary DNA (µg): Lipofectamine™ 2000 (µl) ratios from 1:0.5 to 1:5.

Table I. Suggested starting amounts of reagents for transfection in different culture vessels

Culture vessel	Surface Area per Well (cm^2)	Relative Surface Area (vs. 24-well)	Volume of Plating Medium	DNA (µg) and Dilution Volume (µl)	Lipofectamine™ 2000 (µl)
96 well	0.3	0.2	100 µl	0.2 µg in 25 µl	0.5 µl in 25 µl
24 well	2	1	500 µl	0.8 µg in 50 µl	2.0 µl in 50 µl
12 well	4	2	1 ml	1.6 µg in 100 µl	4.0 µl in 100 µl
6 well	10	5	2 ml	4.0 µg in 250 µl	10 µl in 250 µl
35 mm	10	5	2 ml	4.0 µg in 250 µl	10 µl in 250 µl
60 mm	20	10	5 ml	8.0 µg in 0.5 ml	20 µl in 0.5 ml
100 mm	60	30	15 ml	24 µg in 1.5 ml	60 µl in 1.5 ml

Selecting antibiotic-resistant transfected cells

There are two major benefits for selecting antibiotic-resistant transfected cells:

1. For cells that are very difficult to be transfected or have very low transfection efficiency, using antibiotic selection will kill the cells that were not transfected with the construct. This will be able to reduce the background when analyzing the expression.
2. By using the antibiotic selection, a stable cell line can be established. The stable cell line can be maintained and assessed for the target gene expression for a long-term period.

The following are general procedures for selecting antibiotic-resistant cells.

1. Before doing antibiotic-resistant selection, perform a transient assay to check the protein expression of the construct by Western or ELISA.
2. Following the transfection procedures as outlined in Section V to perform the cell transfection.
3. After 24 hours of transfection, lift the cells from plates using Trypsin-EDTA. Then add G-418 to the medium for selection.
4. The optimal concentration of G-418 is in the range of 50-1500 µg/ml. 100 µg/ml will be a good start point for optimization.
5. Examine the dishes for viable cells every 2 days. Identify the lowest G-418 concentration that begins to give massive cell death for wild-type cells in approximately 7-9 days, and kills all wild-type cells within 2 weeks. Using this concentration to select cells containing the human gene contruct.
6. You can select a mixture of resistant cells or single colony depending on your need.

VII. BACTERIA EXPRESSION PROTOCOL

Transformation

1. Thaw *E. coli* BL21(DE3) competent cells on ice. Pipet 50 µl of BL21(DE3) competent cells into 1.5 ml microcentrifuge tube containing the annealing mixture. Gently mix well and incubate on ice for 30 minutes.
2. Heat shock at 42°C for 45 seconds. Incubate on ice again for two minutes.
3. Add 500 ml LB. Incubate at 37°C with shaking at 250 rpm for one hour.
4. Plate 20 µl and 200 µl of the cells on two LB/Ampicillin (100 µg/ml) agar plates, respectively, and the remaining cells on another plate.
5. Incubate plates overnight at 37°C.
6. Pick single colonies for colony screening.

Minipreps

1. Pick single colonies and make six 2 ml mini cultures with Ampicillin at 100 µg/ml.
2. Incubate at 37°Cwith shaking at 250 rpm for 8 – 12 hours.
3. Transfer 1.5 ml of culture into each of six 1.5 ml microcentrifuge tubes, and spin at 13,000 rpm in a bench-top microcentrifuge for 30 seconds. Save the rest of the cultures at 4°C for future use.
4. Remove and discard supernatent, and use commercial miniprep kits to extract plasmid DNA.
5. Analyze and confirm the plasmid by restriction enzyme digestion. Choose one mini culture for next step, induction and expression.

Induction and Expression

1. Inoculate 1 L LB containing 100 µg/ml of Ampicillin with 250 µl of mini culture.
2. Incubate at 37°C with shaking at 250 rpm.
3. Periodically check the OD_{600} of the culture until the OD_{600} reaches 0.5 – 0.6.
4. Set aside 1 ml of the culture as the un-induced control. Induce the culture with 1 ml of 0.4 M IPTG.
5. Grow the culture at 37°C for 3 hours.

Detection and Purification

1. Remove 1 ml of the induced culture for expression analysis. Pellet the cells by spinning at 13,000 rpm in a bench-top microcentrifuge for 30 seconds.
2. Remove and discard the supernatants and resuspend the cell pellets in 50 µl of ddH_2O. Add 1 µl of PMSF to final concentration of 1 mM to each suspension and then add 50 µl 2 X SDS Sample Buffer. Mix the sample rapidly and boil for 3 to 5 min.
3. Centrifuge in a microcentrifuge for 30 sec. Load 5 µl of the supernatant on a normal SDS-PAGE gel.
4. Run the gel for proper time and stain the gel with Coomassie Blue.
5. After verifying the protein, you can purify the protein using Sigma Anti-FLAG®M1 Agarose Affinity Gel (A4596).

VIII. BACULOVIRUS EXPRESSION

pDream is also a baculovirus transfer plasmid that is compatible with Novagen BacVector® designed for efficient and reliable construction of baculovirus recombinants for protein expression and functional analysis. For the expression of the gene in insect cells, follow the protocols of Novagen BacVector® (http://www.emdbiosciences.com/Products/BrowseProductsByCategory.asp?catid=199) Transfection Kits for optimal performance of the transfer plasmid and the kits.

Order BacVector®-1000 Transfection Kit (Catalog No.: 70059) from Novagen. You can also use BacVector®-2000 Transfection Kit (Catalog No.: 70030) or BacVector®-3000 Transfection Kit (Catalog No.: 70077)

Brief Outlines of Baculovirus Expression Procedures:

1. Use the product as a transfer vector and co-transfect insect cells (SF9 cells) with the BacVector Triple Cut Virus DNA (from Novagen kits).
2. Collect the SF9 cells culture media as recombinant viral stock.
3. Infecting insect cells (SF9 cells) with the recombinant viral stock to produce high level of gene expression.

IX. MOVING GENES TO OTHER VECTORS

The vector, with the gene of interest flanked by *att*B1 and *att*B2 sequences, is compatible with Invitrogen Gateway™ system (https://catalog.invitrogen.com/index.cfm?fuseaction=viewCatalog.viewCategories&pc=110&npc=92&nc=109&). This allows you to move the gene into other expression vectors from Invitrogen if needed. To do so, you need to order the following kits:

1. PCR Cloning System with Gataway® Technology with pDONR™221 or pDONR™/Zeo (Cat. No. 12535-019 or 12535-027)
2. Expression System with Gataway® Technology.
   Cat. No. 11824-026 for *E. coli* expression
   Cat. No. 11827-011 for Baculovirus expression
   Cat. No. 11826-021 for Mammalian expression

A Brief Procedure to Move the Gene of Interest to Invitrogen Expression Vectors:

1. Create an *att*L-containing entry clone by recombination (BP Reaction) of GenScript product (containing *att*B sites) with a donor vector (containing *att*P sites), pDONR™221 or pDONR™/Zeo.
2. Transform appropriate competent *E. coli* to select for and amplify entry clones.
3. Create an *att*B-containing expression clone by recombination (LR Reaction) of entry clone (containing *att*L sites) with a destination vector (containing *att*R sites), from kit with Cat. No. 11824-026 for *E. coli* expression, or kit with Cat. No. 11827-011 for Baculovirus expression, or kit with Cat. No. 11826-021 for Mammalian expression.
4. Transform appropriate competent *E. coli* to select for and amplify expression clones.
5. Express the gene of interest in the appropriate system using the expression clone.

X. cGFP EXPRESSION AS A POSITIVE CONTROL

As a positive control, cGFP gene was cloned into pDream2.1 using Ligation Independent Cloning (LIC) method. cGFP was expressed in all three major protein expression systems: bacteria, insect cells and mammalian cells. The green fluorescence can be directly observed under Fluorescence Microscope. Figure 3 and Figure 4 are the cGFP expression in 293H cells and *E. coli,* respectively.

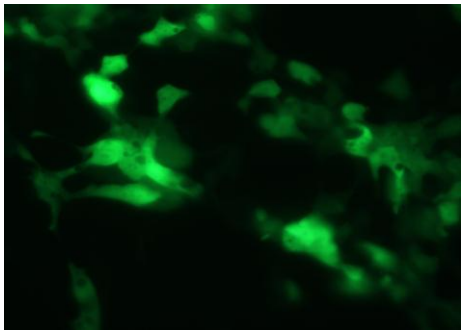


Figure 3. cGFP Expressed in 293H cells

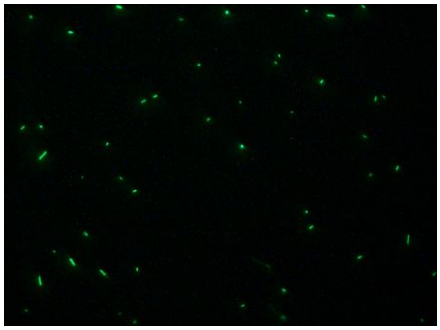


Figure 4. cGFP Expressed in *E. coli*

XI. REFERENCES

1. Aslanidis, C. and de Jong, P. J. (1990) *Nucleic Acids Research* **18**:6069-6074
2. Haun, R. S., Serventi, I. M. and Moss, J. (1992) *Biotechniques* **13**: 515-518

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