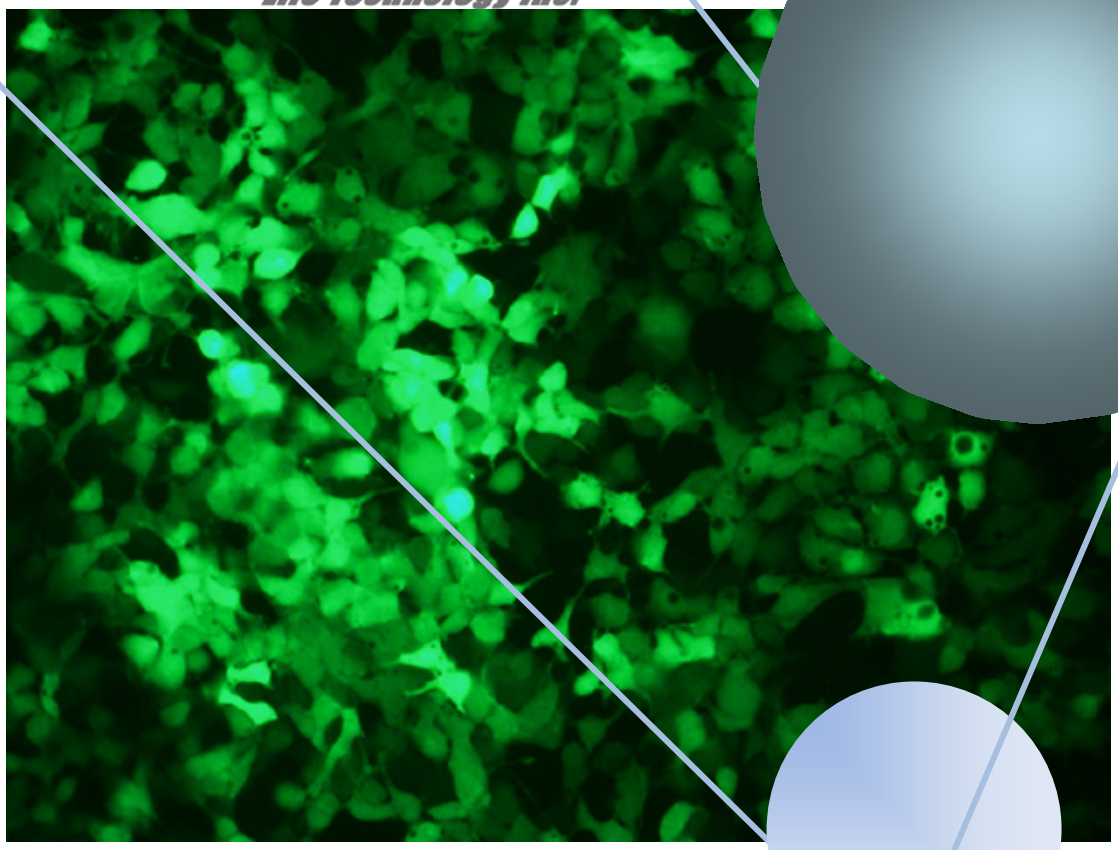


pSLIC-PuroP2A Lentivirus Plasmid Construction Kit

(Cat# LT2002)

[ATCGbio Life Technology Inc. provides pSLIC-PuroP2A lentivirus plasmid construction kit to let user create the lentivirus plasmid expressing the gene of interest. This kit comes with SLIC enzyme and a lentivirus plasmid which contains puromycin resistance gene and P2A sequence.

SLIC (sequence and ligation independent cloning) allows users to insert PCR product into the plasmid without ligation. P2A peptide sequence allows to express both puromycin resistance gene and user's gene of interest by a single CMV promoter (bicistronic).

Our proprietary SLIC enzyme system which has superb performance and temperature stability (stable even at room temperature for a few days).]

Contents

FOR RESEARCH USE ONLY
Not for use in clinical or diagnosis purpose
Important Notice:
Laboratory workers handling pathogenic lentiviruses, recombinant lentiviral vectors, naturally or experimentally infected laboratory animals, or clinical specimens potentially infected with lentiviruses. Diagnostic specimens that contain human blood, body fluids or tissues can be handled and manipulated at the BSL-2 level. BSL-2 is also appropriate for generating and using lentiviral vectors, and handling animals and animal tissues, blood, body fluids and cell lines that are infected with lentivirus vectors. When you practice recombinant lentiviral vectors, please following the requirements outlined in http://oba.od.nih.gov/rdna_rac/rac_guidance_lentivirus.html and *CDC Biosafety in Microbiological and Biomedical Laboratories*, 5th edition, the *NIH Guidelines for Research Involving Recombinant DNA Molecules*, latest edition.

pSLIC-PuroP2A Lentivirus Plasmid Construction kit (LT2002)

Introduction

ATCGbio Life Technology Inc. provides pSLIC-PuroP2A lentivirus construction kit to let user create the lentivirus plasmid expressing the gene of interest. This kit comes with SLIC enzyme and a lentivirus plasmid which contains puromycin resistance gene and P2A sequence. SLIC (sequence and ligation independent cloning) allows users to insert PCR product into the plasmid without ligation. This technique has been developed last 10 years (for example, see Nature Methods 4:251-256, 2007) and we created our proprietary SLIC enzyme system which has superb performance and temperature stability (stable even at room temperature for a few days). P2A peptide sequence allows to express both puromycin resistance gene and user's gene of interest by a single CMV promoter (bicistronic).To make the ligation effective, a staffer sequence was inserted between EcoR V. User first digests the plasmid with EcoR V and then inserts the PCR product by SLIC enzyme reaction. User performs PCR (gene of interest) with primers having 15bp homologous to the ends of EcoR V sequence. In 30 min reaction with SLIC enzyme, the PCR product is inserted downstream of P2A sequence.

P2A-like peptide sequence was discovered in various virus genomes and found to self-cleave near the end of sequence during translation. P2A sequence in our plasmid is GSGATNFSLLKQAGDVEENPG/PD. The first part (before slash "/") GSGATNFSLLKQAGDVEENPG is attached to puromycin resistance gene product and the second part (after slash) PD is attached to the gene of interest users insert. Start codon of gene of interest (ATG) may be removed. Alternatively, user can put flag-tag (DYKDDDDK) sequence at the N-terminus gene of interest just by a single PCR as shown in this manual.

The kit contains enough plasmids and reagents to perform at least 20 reactions for creating lentiviral vector plasmids.

Kit Components and Storage Conditions

(shipped at RT, and stored at following temperature)

Components	**Size and Storage conditions**
pSLIC-PuroP2A plasmid	20µl, 200ng/µl, 4°C **This plasmid is not intended to amplify.**
10 × SLIC enzyme Solution	25µl, -20 °C (enzyme is stable at least 4 days at room temperature)
GFP template DNA (Control)	10 µl, 10ng/µl, 4°C
GFP forward and reverse primer mixture	10 µl, 10 µM each, 4°C

User Provided Materials

1. Chemically Competent *E. Coli.*
   We recommend to use our SLIC-easy *E.Coli* (SLIC-1001) to obtain the best result. We also confirmed that Top10 cell (Invitrogen) works well. We don't recommend DH5alpha.
2. EcoRV endonuclease. We used New England Biolab product.

3. Proof reading PCR kit. We recommend Takara PrimeStar HS (Cat# R010A). Non-proofreading PCR enzyme such as Taq should not be used.
4. PCR gel-purification kit. Such a kit is available from Qiagen, Clontech and others.
5. PCR primers: It will be 35-40nt in length. De-salt grade or higher quality is satisfactory.

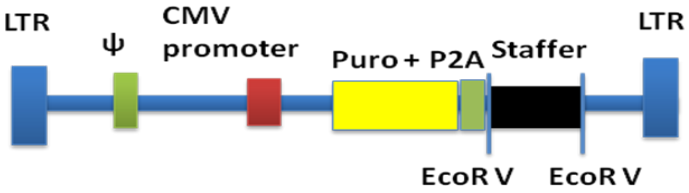
Plasmid Structure and Primer Design

Gene of interest should be subcloned by PCR from other vector by primers containing 15bp homologous to end of EcoR V digestion site (see below figure). Red colored sequences need to be incorporated into PCR product.

The PCR product should be gel-purified. By SLIC enzyme reaction, PCR fragments can be inserted to pSLIC-PuroP2A plasmid in 30 minute.

Our pSLICs plasmid size is 6.8kb containing 3.4 kb basic lentivirus components between 2 LTRs. Original 5' LTR promoter activity will be self-inactivated during virus production. pSLIC-PuroP2A plasmid has puromycin resistance gene with P2A sequence (total ~0.7 kB) followed by a staffer sequence flanked by EcoR V sites.

pSLIC-PuroP2A Structure



5'- TGGAGGAGAACCCTGGACCTGAT –Staffer –ATCCAGCACAGTGGCGGCCGCTCGAC-3'

Guideline to Design the PCR Primers

By PCR subcloning, PCR product (gene of interest) contains15bp plasmid homologous sequences (red colored sequence above). The following steps are the guideline for primers design.

1. To design gene-specific part of the primers, use base-stacking calculated Tm.
   This is one of websites to do calculation.
   http://www.promega.com/techserv/tools/biomath/calc11.html
   In the calculator,
   a. Enter primer sequence (not include the red colored sequence above) in Step 1
   b. Check "Adjust Mg Concentration" in Optional of Step 2
   c. Get the result from the "base-stacking calculated T_m"
   (Parameters: K^++Na^+=50mM, Mg^{++}=1.5mM, Click "Adjust Mg Concentration")

2. Forward primer.

AACCCTGGACCTGAT + gene specific 5' sequence (ATG can be removed).
Tm of gene specific 5' sequence should be around 60-65 °C.

3. Reverse primer
   Firstly design like this:
   Gene specific 3' sequence (stop codon should be included) + ATCCAGCACAGTGGC
   Tm of gene specific 3' sequence should be around 60-65°C.
   Then, order antiparallel (reverse + complement) of this sequence.

Design primers for Creating GFP Control Plasmid

(The control GFP primers and template come with the kit)

Here is the example to insert GFP sequence in primers (see declaimer in page 8).
The control PCR primers which come with kit were designed as follows,
GFP sequence is
5'- ATGGTGAGCAAGGGCGAGGAGCTGT.....TGGACGAGCTGTACAAGTAA -3'

1. Choose 15nt (skip ATG) and 20nt in the GFP sequences for forward and reverse primers respectively, and calculate Tm.
   5' 15nt: GTGAGCAAGGGCGAG Tm= 60°C
   3' 20nt: TGGACGAGCTGTACAAGTAA Tm= 62°C

2. Design PCR primers for SLIC reaction
   Forward primer: AACCCTGGACCTGAT + GTGAGCAAGGGCGAG
   Reverse primer: TGGACGAGCTGTACAAGTAA + ATCCAGCACAGTGGC (Before antiparallel)
   Reverse primer: GCCACTGTGCTGGATTTACTTGTACAGCTCGTCCA (After antiparallel)

Step By Step Protocol

1. **Preparation of digested pSLIC-PuroP2A plasmid by EcoR V**
   Before doing SLIC reaction, the pSLIC-PuroP2A needs to be linearized by EcoR V. This step can do conveniently on thermal cycler.
   - Take pSLIC-PuroP2A plasmid 15µl (3µg) for EcoR V digestion in 30µl final volume and then heat-inactivated.
   - After digestion, check digested products (3-4 µl) by running NDA gel. It should produce 2 fragments in size of 2kb (staffer) and 7-8kb.
   - **The final concentration of digested pSLIC-PuroP2A plasmid is 100ng/µl.**
   - Keep it at -20°C until use. Repeated freeze-thaw does not affect performance.

   (Note: read the instruction coming with the enzyme for conditions of digestion and inactivation. In case of New England Biolab's EcoR V, reaction should be 15µl pSLIC-PuroP2A plasmid + 11 µl water

+3 µl Digestion Buffer +1µl (20 U) EcoRV at 37°C for 1hr and inactivation at 80°C for 20min.**)**

2. PCR and SLIC reaction

PCR for the target sequence with designed primers

In case of Takara PrimeStar HS (Cat# R010A), 50 µl volume of PCR will be:

5x buffer	10µl
dNTP mix	4µl
10 µM forward primer*	1.5µl (final concentration 0.3µM)
10 µM reverse primer*	1.5 µl (final concentration 0.3µM)
20ng/µl template	1 µl(amount of template should be 20-40ng)
nuclease free water (NFW)	31.5µl
PrimeStar HS enzyme	0.5µl

* In case of Control GFP amplification, add 1.5 µl mixture which contains both forward and reverse primers

Processing speed of this PCR enzyme is 1-2 kb/min. Adjust extension time accordingly. In case of GFP, 40 seconds should work.

PCR conditions:

1). 98 °C 2min
2). 98 °C 10 seconds
3). 55 °C 5 -10 seconds
4). 72 °C 40 seconds (repeat step 2-4, total 24 cycles)
5). 72 °C 3 min
6). 4°C Hold

3. Gel purify PCR product

Run the PCR product on EthBr or SYBR safe impregnated 1% DNA gel and cut the expected size of band. Gel purify by PCR gel-purification kit with 20-30µl elution volume. Measure DNA concentration with Nano-drop or spectrophotometer.

4. SLIC reaction

Set thermal cycler as following parameters.

1). 20 °C 10 min
2). 70 °C 10 min
3). 50 °C 10 min
4). 4.0 °C hold

Note: Molar ratio of PCR insert and pSLIC vector should be 5:1 ratio.
Digested pSLIC vector is 100ng (in 1µl), 8 kb size,
If PCR product size is 1kb, it requires 1/8 x 5 x100 = 63 ng
If PCR product size is 3 kb, it requires 3/8 x 5 x100 = 188 ng.

Set up one PCR tube at room temperature and add:

PCR product **+** Water: 8 µl **(PCR product volume is calculated by molar ratio with pSLIC vector)**
pSLIC plasmid (EcoRV digested): 1 µl
10x SLIC enzyme: 1 µl (the enzyme should be added lastly).

Pipet up-down to mix

Start the thermocycler and confirm temperature to have reached at 20°C.
Then immediately put the tube on the thermocycler.

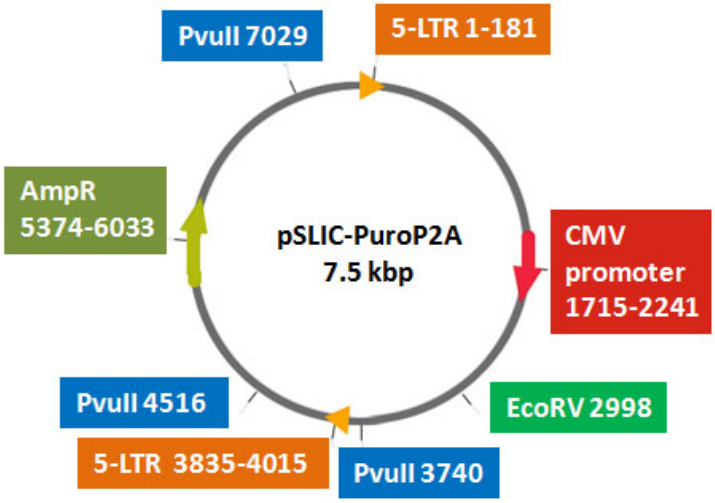
5. Transform *E.Coli* and preparation of plasmid mini-prep

After SLIC reaction finish, centrifuge briefly. Transform chemically competent *E.Coli* (we recommend our SLIC-Easy *E.Coli, SLIC-1001*) with 3µl reaction. After heat-shock, incubate with SOC medium for 1hr, spread entire content on Ampicillin(100 µg/ml) agar plate and incubate it for overnight (ideally 32-34°C*). Pick up 3 colonies and incubate in each 2ml LB overnight (ideally 32-34°C*).

*Lentivirus plasmid is most stable in *E.Coli* growing at 32-34 °C. Higher temperature frequently results mutation (see FAQ of Amplify Viral Plasmid in our website).

6. Confirm insert by PvuII digestion

First, confirmation can be done by PvuII digestion (we recommend PvuII-HF from New England Biolab). Typically at least one in 3 colonies is the right plasmid. If not, pick up more colonies.



1). As seen the plasmid map** above, it produces 2 fixed length fragments
2.5 kb - From PvuII [4516] to PvuII [7029]
0.75 kb - From PvuII [3740] to PvuII [4516]

2). Other fragment is depended on the insert at EcoRV[2998].
- If there is no insert, it will be ~4.2 kb (PvuII[7029] to PvuII[3740])
- If the insert size is 2 kb and does not have any PvuII digestion site, it will be 4.2 kb+2kb =6.2 kb.
- If the insert size is 2 kb and cut by PvuII at 0.5kb from 5', it will produce 2 fragments.
  a. 3.5 kb (PvuII[7029] to EcoRV[2998]+ 0.5 kb (insert 5') = 4 kb
  b. 1.5 kb (insert 3') + 0.75 kb (EcoRV[2998] to PvuII [3740]) = 2.25 kb

3). Precise sequence can be analyzed by sequencing. We use first 20bp of P2A peptide **GGAAGCGGAGCTACTAACTT** as a sequence primer.
The expected result should be:

GGAAGCGGAGCTACTAACTTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGAG
AACCCTGGACCTGAT + 5'-insert

**The map was created with compilation of sequences and restriction enzyme digestion. Sequence analysis was not performed in entire vector.

7. Amplification of plasmid.

Our SLIC-Easy *E. Coli* (SLIC-1001) is suitable for further amplification (Midi to Max) as far as at 32-34 °C for amplification. In rare, it may spontaneously produce mutation (3-5 kb size). In such a case, we recommend to use VP-Easy *E.Coli* (VP-1001) or other *E.Coli* suitable for viral plasmid amplification.

8. Production of lentivirus.

We recommend to use our lentiviral production kit (LT1001) to produce lentivirus. Although precise virus titer depends on the insert length and structure, in case of 1kb or 3kb insert, 200 µl or 500 µl of crude virus is sufficient to infect more than 90% of Human endothelial cells seeded at 30%. See detailed information in the kit manual.

9. Expected results

In most of cells, it takes 1-3 days to express proteins. Expression by CMV promoter is typically high and fast in human cells but low and slow in mouse cells. Therefore puromycin should be added at the day 3 after infection. Concentration should be determined empirically. Better start with lower concentration in mouse cells. If expression is high, fastest way to choose resistance cells is to add puromycin (0.5µg -1µg/ml) overnight and re-plate the cells next day in presence of 1µg/ml puromycin. By doing so, non-infected cells do not attach on the plate and almost all the proliferating cells are resistant.

Appendix

Creation of N-terminus Flag-tagged Gene of Interest

Our P2A sequence end CCTGAT (PD in amino acid) is going to attach to 5' of gene of interest. Typical flag sequence is GATTACAAGGATGACGACGATAAG (DYKDDDDK) and the first GAT of flag sequence is the same as the last P2A sequence.

Thus, Forward primer to create N-terminus flag-tagged gene of interest with SLIC reaction will be: AACCCTGGACCTGATTACAAGGATGACGACGATAAG (36nt) + 5'- sequence of gene of interest (ATG can be skipped).

Length of 5'- sequence of gene of interest will be between 17-25nt. The entire length of primer becomes 53 -61nt. If this length of primer is ordered, it is typically recommend to do PAGE purification procedure which is very expensive.

We can avoid this problem by splitting entire sequence into two primers.
Forward 1: GATGACGACGATAAG (last 15nt of flag sequence) + 5'-gene of interest
Forward 2: AACCCTGGACCTGATTACAAGGATGACGACGATAAG + 6nt of 5'-gene of interest.
Forward 1 will be about 40nt and forward 2 will be 42nt which can be ordered de-salt or one more higher grade. Two sequences are overlapped by 21nt whose Tm becomes more than 55°C.

In PCR reaction, do 2-step continuous PCR showing as the follows.
First, add forward 1 primer at 0.03µM and reverse primer 0.3µM in 50µl reaction.
Then program thermal cycler like this

98 °C 10 sec
55 °C 5 sec
72 °C 1 min/kb Repeat 8 cycles
Hold at 80 °C <=add forward 2 primer at 0.3 µM then resume to next PCR
98 °C 10 sec
55 °C 5 sec
72 °C 1 min/kb Repeat 20 cycles
72 °C 3 min (clean up)

By doing this, flag-tag containing gene of interest is ready for SLIC reaction.

Disclaimer

We provide GFP sequence as PCR template. Because of nature of PCR, we do not guarantee the function of the sequence.

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