Cloning ecGReg, bpeGReg or truncated genes into the pTrc99A or pET-3a vectors

Xuehua Wan, Jennifer A. Saito, Shaobin Hou, Maqsudul Alam

Abstract

The protocol describes a molecular cloning method in our laboratory.

Citation: Xuehua Wan, Jennifer A. Saito, Shaobin Hou, Maqsudul Alam Cloning ecGReg, bpeGReg or truncated genes into the pTrc99A or pET-3a vectors. **protocols.io**

dx.doi.org/10.17504/protocols.io.ijxccpn

Published: 06 Jul 2017

Protocol

DNA Amplification

Step 1.

The *ecGReg*, *bpeGReg*, or truncated genes were amplified by PCR (polymerase chain reaction). The PCR reactions were set up as below, with PfuTurbo DNA polymerase being added after a 2 minute hot start at 94 °C. This was followed by 25 or 30 cycles of 94 °C for 30 seconds, 55 °C (varies from 45 °C to 62 °C according to the Tm of primers) for 30 seconds, and 72 °C for 1-3 minutes with a final extension at 72 °C for 7 minutes. PCR products were analyzed by agarose gel electrophoresis.

DNA template	1 µl
10 × Pfu Buffer	5 µl
dNTPs (10 mM)	1 µl
DMSO	2 µl
Primer 1 (10 pmole/µl)	1 µl
Primer 2 (10 pmole/µl)	1 µl
ddH2O	38 µl
PfuTurbo DNA polymerase (2.5U/µl)	1 µl

PCR Purification

Step 2.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen).

The 50 µl PCR product was mixed with 250 µl of Buffer PB, incubated at room temperature for two minutes, applied to a QIAquick spin column, and centrifuged for one minute at 14,000 rpm. The flow-

through was discarded and the bound DNA was washed twice with 750 µl Buffer PE. After the flow-through was discarded, the column was centrifuged for additional one minute to remove residual ethanol. The QIAquick column was placed in a clean microcentrifuge tube and air-dried for 20 minutes. To elute, 30 µl of ddH2O was added to the center of the QIAquick membrane, allowed to stand for two minutes, and then centrifuged for one minute at 14,000 rpm.

TOPO Cloning

Step 3.

The purified blunt-end PCR product was mixed with pCR4Blunt-TOPO vector as below, and then incuated for 5-30 minutes at room temperature.

AMOUNT
1 µl Additional info: TOPO vector
AMOUNT
3 µl Additional info: ddH2O
AMOUNT
1 µl Additional info: PCR product

Transformation of Escherichia coli Competent Cells

Step 4.

The TOPO cloning reaction was mixed with 25 µl of TOP10 or Mach1-T1^{R} competent cells, and then incubated on ice for 20-30 minutes. The cells were heat shocked at 42 °C for 45 seconds and then placed on ice for two minutes. Then, 200 µl of LB medium was added to the cells and incubated at 37 °C for one hour with shaking. The cells were centrifuged at 5,000 rpm for two minutes. The cell pellet was resuspended in about 50 µl of LB medium and spread on 1.2% agar plates containing the appropriate antibiotics. The plates were incubated at 37 °C for 10-16 hours.

Plasmid Isolation

Step 5.

Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen). Single colonies were inoculated into 2 ml of CircleGrow medium and incubated overnight at 37 °C with shaking. The cells were harvested by centrifugation and the cell pellet was resuspended with 250 µl of Buffer P1. Then, 250 µl of Buffer P2 was added and the tubes were gently inverted for 4-6 times to mix. After 5 minutes, 350 µl of Buffer N3 was added and mixed thoroughly by inverting the tubes. The tubes were incubated at 4 °C for 10-15 minutes and then centrifuged at 14,000 rpm for 10 minutes at 4 °C to remove the precipitated cell debris, proteins, and genomic DNA. The supernatant was transferred to clean tubes and centrifuged at 14,000 rpm for 15 minutes at 4 °C. The supernatant was transferred to QIAprep spin columns, centrifuged at 14,000 rpm for one minute at room temperature, and the flow-through was discarded. The columns were washed twice by adding 750 µl of Buffer PE and centrifuging for one minute. After the second wash, the column was centrifuged for an additional one minute, transferred to a clean microcentrifuge tube, and air-dried for 45 minutes. The plasmid DNA was eluted with 35 µl of Buffer EB.

Restriction Enzyme Digestion

Step 6.

Restriction enzyme and alkaline phosphatase were purchased from Promega. The preparative

digestion reaction was set up as below and incubated in 37 °C water bath for one hour. The vector was dephosphorylated by adding 1 µl of alkaline phophatase (1U/ µl) and incubating at 37 °C for an additional one hour. This was done to prevent self-ligation of partially digested vector.

AMOUNT
1 µl Additional info: enzyme(s) (10U/µl) each
AMOUNT
3 µl Additional info: 10X enzyme buffer
AMOUNT
21 µl Additional info: ddH2O
AMOUNT
5 µl Additional info: DNA

Extraction of DNA from Agarose Gels

Step 7.

DNA was purified from agarose gels using the Geneclean Spin Kit (Qbiogene). The digested DNA was run on an agarose gel and the desired bands of DNA were cut out and placed in a Geneclean Spin filter. Then, 400 µl of Geneclean Spin Glassmilk was added to the filter and heated at 55 °C to melt the gel, mixing every two minutes. The tube was centrifuged at 14,000 rpm for one minute and the flow-through was discarded. Then 400 µl of ice-cold Geneclean Spin New Wash was added to the filter, centrifuged at 14,000 rpm for one minute, and the flow-through was discarded. This washing step was repeated 3 more times. The filter was centrifuged for an additional one minute, transferred to a new catch tube, and heated at 55 °C to remove residual ethanol (until Glassmilk powder became loose). To elute, 15 µl of ddH2O was added to the filter, allowed to stand for 1-2 minutes, and then centrifuged for one minute.

Ligation to pTrc99A or pET-3a vectors

Step 8.

The DNA purified by Geneclean was ligated overnight at 14 °C as the below.

AMOUNT
1 µl Additional info: 10X T4 DNA ligase buffer
AMOUNT
1 µl Additional info: T4 DNA ligase
AMOUNT
8 µl Additional info: DNA

Repeat Transformation and Plasmid Isolation

Step 9.

Repeat steps 4 and 5.