2019/10/28

Instruction for iVEC Express

iVEC Express is an *E. coli* strain for in vivo cloning and subsequent protein expression. It expresses T7 RNA polymerase, enabling gene expression of interest from the T7 promoter. You can clone the DNA fragment in *E. coli* cells in the same way as the iVEC3 strain, and then continuously proceed to protein expression of the cloned gene.

There are 2 types: iVEC express (lac), in which the T7 RNA polymerase gene (T7gene1) is introduced downstream of the lac promoter by replacing the lacZ gene on its chromosome, and iVEC express (ara), in which the T7gene1 is introduced downstream of the araBAD promoter by replacing the araBAD genes.

Please refer to the following paper for iVEC:
Exonuclease III (XthA) enforces in vivo DNA cloning of Escherichia coli to create cohesive ends. Nozaki and Niki, J. Bacteriol., doi: 10.1128/JB.00660-18.

Genotype of iVEC strain

iVEC express (lac): MG1655 ΔhsdR ΔendA ΔlacZ::T7gene1
iVEC express (ara): MG1655 ΔhsdR ΔendA ΔaraBAD::T7gene1

Inhibition of the gene expression by the lacI repressor

In iVEC express (lac), expression of T7gene1 is not completely repressed. Therefore, the cloned gene is expressed from the T7 promoter even in a medium that does not contain IPTG. To solve this problem, it is recommended to grow the strain in a medium containing glucose. In glucose-containing media, expression of T7 RNA polymerase is repressed, thereby preventing leakage of gene expression from the T7 promoter. Using this property, gene expression can be induced by transferring the cells from medium containing glucose to that not containing glucose, without using IPTG. Although there might be a case that cells do not grow if a toxic gene to E. coli cell is cloned, leaky expression from the T7 promoter does not usually cause a problem when grown in a glucose-containing medium.

Induction of protein expression by arabinose

In iVEC express (ara), L-arabinose can induce gene expression. There is a little leakage of gene expression from the araBAD promoter in media that do not contain L-arabinose. The leakage is also repressed in a glucose-containing medium, so it is recommended that glucose is added to the medium when a gene toxic to E. coli cell is cloned.

Notes

Synthetic DNA primers for PCR should be designed to have homologous sequences with about 20 ~ 40 bp to the vector.

An example of primers used to clone the gfp gene into the pET28a vector is shown in Fig 1.

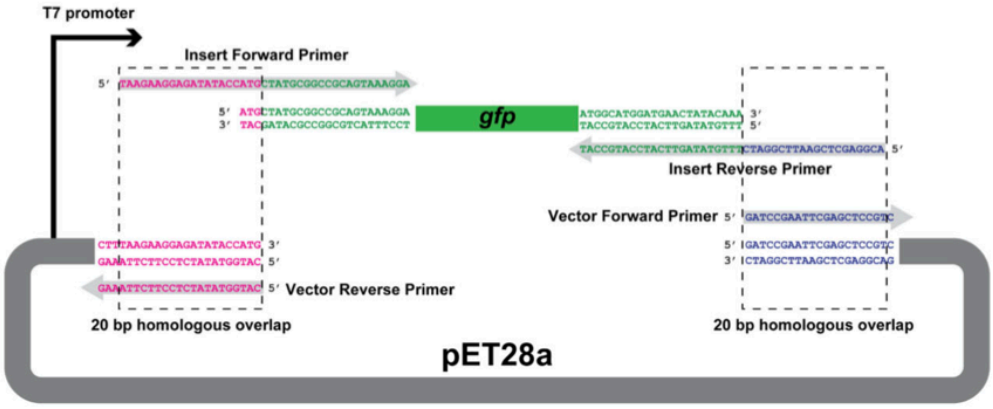


Fig 1. cloning the gfp gene into the pET28a vector

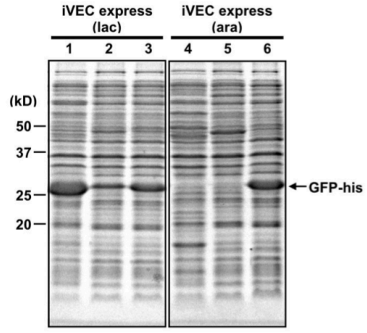
Note that building large plasmids, such as those larger than 10 kb, may not be successful due to reduced efficiency of DNA fragments incorporation. In that case, it may be solved by increasing the amount of the DNA to be introduced or lengthening the homologous sequences. The iVEC express strain carries a wild-type recA gene to prevent growth rate reduction. Thus, the recombination reaction may dimerize the plasmid in the cell without affecting protein expression.

Transformation of iVEC strains

In vivo cloning in the iVEC strain performs its best by the TSS method. Transformation by the TSS method described below is very simple; it can be carried out in a single tube from the production of competent cells to transformation.

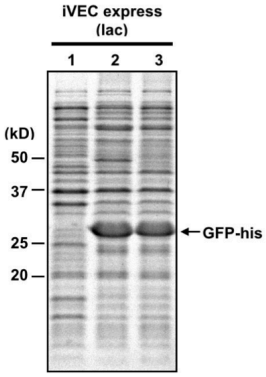
An example of an experiment

A representative result of expressing GFP-his from the pET28a vector in the iVEC express strain is shown on the right (Figure 2). In iVEC express (lac), a large amount of GFP-his was expressed in the overnight culture without IPTG (Figure 2, Lane 1). This indicates that even in the absence of IPTG, the T7 RNA polymerase induces expression of the gene encoding GFP-his under the control of the T7 promoter. On the other hand, iVEC express (ara) showed little expression of GFP-his in the medium without arabinose (Figure 2, Lanes 4 and 5), and GFP-his was expressed only in the medium containing arabinose (Figure 2, Lane 6). Leakage of expression under non-induced conditions in the iVEC express (lac) strain was significantly repressed by the addition of 0.5% glucose to the medium (Figure 3, Lane 1). After transferring the cells grown overnight from glucose-containing medium to glucose-free medium, the GFP-his was expressed as equivalent levels as those induced by IPTG (Figure 2, Lane 1 and Figure 3, Lane 2).



1 - 3: iVEC express (lac)
4 - 6: iVEC express (ara)
1, 4: Over night culture in L medium
2, 5: Non-induced culture in L medium
3: Induced culture in L + 0.5 mM IPTG
6: Induced culture in L + 0.2 % L-arabinose

Fig 2. GFP-his protein expression using iVEC express strain



1: Overnight culture in L + 0.5 % glucose
2: Culture transferred to L medium without glucose
3: Induced culture in L + 0.5 mM IPTG

Fig 3. Repress the leakage of expression by glucose in iVEC express

Transformation of iVEC

Reagent

- TSS (Transformation and Stock Solution) 50 ml (about 50 transformation)

L broth 25 ml

2xTSS 20 ml

DMSO 5 ml

Store at 4 ºC.

- 2xTSS (20 ml)

PEG8000 4 g

1M MgSO4 2 ml

80 % glycerol 5 ml

Add L broth to 20 ml

Autoclave for 15 min at 120 ºC.

Mix well before use.

Store at 4 ºC.

- L broth (1,000 ml)

Tryptone 10 g

Yeast Extract 5 g

NaCl 5 g

Add H2O to 1,000 ml

Adjust to pH 7.0 with 5N NaOH.

Autoclave for 15 min at 120 ºC.

Preparation of DNAs for transfromation

・ 15 - 40 bp of the homologous sequences at the end of insert and vector are required. The longer the homologous sequence, the greater the number of transformed colonies.

・ Using 100 ng of PCR products are enough. However, when introducing the longer DNA which exceeds 5 kb, the more amount of the DNA may be required.

・ You can use PCR products without purification, if the template vector DNA in PCR reaction is less than 50 pg/μl.

・ If the amount of the template vector DNA can't be reduced, treatment of the template vector DNA with DpnI after PCR will increase the proportion of positive clones.

Preparation of competent cells and transformation

Day 1: Preparation of competent cells (Required time: 3 min)

- L broth
- Sterilized tooth pick or sterilized tip
- Incubator at 37°C

1. Pick a colony on an agar plate or glycerol stock of iVEC strain using a sterilized toothpick. (A little is just fine.)
2. Suspend cells in a 1.5 ml tube that is filled with 1 ml of L broth.
3. Stand the tube overnight (about 20 hours; 16 - 24 hours are also fine) at 37 ºC.

Day2: Transformation（Required time: 80 min, Actual working time: 5 min)

- Ice bath
- Cooled centrifuge
- Water bath incubator at 37°C
- Liquid nitrogen
- Linearized vector and insert DNA amplified by PCR. Purification of the PCR products is not essential.

1. Prepare 100 μl of ice-cold TSS (Transfromation and Stock Solution) medium in a 1.5ml tube. Add 1 - 2 μl (100 ng DNA) each of PCR-amplified insert and vector DNAs into the TSS medium and mix well.
2. Chill the overnight culture in 1.5 ml tube on ice for 5 - 10 min.
3. Centrifuge the tube at 5,000 g for 1 min at 4 ºC.
4. Remove the supernatant and stand it on ice.
5. Resuspend the cells with TSS-DNA solution by pipetting.
6. After resuspension, immediately freeze the tube with liquid nitrogen.

※Important; Freezing the tube in liquid nitrogen is recommended. Freezing the tube in -80 or -30 ºC reduces the number of transformants.

7. Put the frozen tube on ice and incubate it for 10 min on ice.
8. Shortly mix the tube by vortex (for 1 sec) and put it back on ice and incubate for further 10 min on ice.
9. Add 1 ml of L broth (at room temperature) and mix it by inversion.
10. Incubate the tube in a water bath incubator at 37ºC for 45 min. (Heat shock is not needed.)

11. Centrifuge it at 5,000 g for 1 min to spin down the cell. Remove 1 ml of the supernatant and resuspend the cells with 100 μl of the remained supernatant.
12. Spread the cells on an agar plate with appropriate antibiotics and incubate it at 37ºC for overnight.
13. The growing colonies can be used to check for expression after insertion of the insert has been confirmed by colony PCR.

Protocol for preparation of freezing stock competent cell

Reagents

● 2xTSS (20 ml)

PEG8000 4 g

1M MgSO4 2 ml

80 % glycerol 5 ml

Add L broth to 20 ml

Autoclave at 120 ºC for 15 min.

Mix well before use.

Store at 4 ºC.

● DMSO

Preparation of DNAs for transfromation

・ 15 - 40 bp of the homologous sequences at the end of insert and vector are required. The longer the homologous sequence, the greater the number of transformed colonies.

・ Using 100 ng of PCR products are enough. However, when introducing the longer DNA which exceeds 5 kb, the more amount of the DNA may be required.

・ You can use PCR products without purification, if the template vector DNA in PCR reaction is less than 50 pg/μl.

・ If the amount of the template vector DNA can't be reduced, treatment of the template vector DNA with DpnI after PCR will increase the proportion of positive clones.

Preparation of competent cells

Day 1

1. Pick colony on an agar plate or glycerol stock of iVEC strain using a sterilized toothpick into 3 ml of L broth.
2. Incubate with shaking at 37ºC for overnight (about 16 hours).

Day 2

1. Add 1 ml of overnight culture into 60 ml of L broth preheated to 37ºC and incubate for 90 min. Usually, after 90 min incubation, OD600 becomes 0.4 - 0.6.
2. Stand the flask on ice for 5 - 10 min. From this step, keep the cells on ice.
3. Centrifuge at 5,000 g for 5 min at 4 ºC and discard the supernatant.
4. Resuspend the cells with 2 ml of ice-cold L broth.
5. Add 1.6 ml of ice-cold 2xTSS and mix.
6. Add 0.4 ml of DMSO (room temperature) and mix.
7. Aliquot 0.1 ml into sterilized microcentrifuge tubes.
8. Freeze the tubes with liquid nitrogen and store at -80 ºC.

Transformation

1. Melt the frozen competent cells on ice.
2. Add 1 - 2 μl (100 ng DNA) of PCR products of insert and vector into competent cell and mix.
3. Incubate on ice for 20 min.
4. Add 1 ml of L broth (room temperature) and mix by inversion.
5. Incubate the tube in a water bath incubator at 37ºC for 45 min.
6. Centrifuge at 5,000 g for 1 min to spin down the cell. Remove 1 ml of the supernatant and resuspend the cells with 100 μl of the remaining supernatant.
7. Spread the cells on an agar plate with appropriate antibiotics, and incubate at 37ºC for overnight.
8. The growing colonies can be used to check for expression after insertion of the insert has been confirmed by colony PCR.