pORF-CLONE Vector DNA

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Contents

This vector system has been developed at the Max-Planck-Institute for Molecular
Genetics, Berlin, Germany and the Technical University, Berlin, Germany.

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pORF-CLONE Vector System

Vectors for improved cloning of open reading frames in yeast

1. Summary

The yeast shuttle/expression vector pORF-CLONE has been specifically developed for an improved and facilitated selection of cloned cDNA inserts containing open reading frames (ORFs) thus allowing an enriched growth of clones expressing authentic polypeptides. This vector system is particular useful for the development ofa a high-throughput technique for the one-step generation of high-quality cDNA libraries in the yeast *Saccharomyces cerevisiae* and a direct, time-saving screening of random-primed cDNA libraries.

In brief, the selection system is based on the *HIS3* marker gene fused to the C-terminus of the cDNA insert. The cDNAs cloned in-frame result in histidine prototrophic yeast cells growing on minimal medium, where as clones bearing the vector without insert or out-of-frame inserts should not grow on this medium.

2. Introduction

In recent years, the genome sequencing projects (e.g. the human genome project) have generated enormous amounts of sequence data which have to be processed and elucidated. The identification and understanding of the function of the newly discovered genes has now become an urgent challenge. Recently, high-throughput technologies have been developed that allow the monitoring of gene activity on the transcriptional level by analysis of complex expression patterns of a specific tissue (Harrison, S.M. *et al.,* 1995; Schena, M. *et al.,* 1996; Perret, E. *et al.,* 1998). The next step is the profiling of protein products encoded by expressed cDNA clones to obtain more information on their regulation, biochemical function, and potential interaction partners. This requires the simultaneous expression of protein from a large number of cDNA clones, which has been performed in the *Escherichia coli* bacterial system (Büssow, K. *et al.,* 1998).

When screening cDNA expression libraries for clonal protein expression (e.g. in high-throughput on automatically gridded high-density protein arrays), the unselected frequency of in-frame clones in such libraries with random orientation is statistically only 1/6 of all clones. Thus, a vector that enables the direct selection of open reading frames (ORFs), improving the yield of clones expressing protein, would be extremely useful.

Previously, *E. coli* vectors have been constructed on the basis of the β-galactosidase coding sequence for the generation of in-frame fusion libraries (Gray, M.R. *et al*., 1987). However, only DNA fragments in the range of 100 - 1000 bp could be enriched using this system. Moreover, expression of the marker gene was also observed when the *lacZ* gene was not in frame with the cDNA because of the polycistronic mRNA in prokaryotes and the reinitiation of translation. Davis and Benzer (Davis C. A. *et al.,* 1997) constructed a vector that confers kanamycin resistance to the host on translation of an insert in the correct reading frame. They made three size-fractionated cDNA libraries in *E. coli*, namely 100 - 200, 200 - 300, and 300 - 500 bp. Only the library that contained small cDNAfragments in the range of 100 - 300 bp could be enriched to 60% - 80% for ORF clones.

For improving the selection efficiency, especially of larger inserts, it would be quite advantageous to use an eukaryotic host, which, in contrast to *E. coli*, has a codon usage homologous to that of mammalian cells, thus avoiding frameshifts as well as translation and post-translational modification problems. Therefore, the eukaryotic yeast *Saccharomyces cerevisiae*, which is able to produce soluble proteins in large amounts (Romanos, M.A. *et al*., 1992) is a suitable host for expressing functional, correctly modified proteins.

With the pORF-CLONE vector a system has been developed in which a C-terminally fused marker gene is expressed only if the cloned insert carries no internal stop sequences, which may result from frameshifts or 5' and 3' untranslated regions. Consequently, the use of random-primed cDNAs is required in this system instead of oligo (dT)-tailed cDNAs that carry their own C-terminal termination codon (Holz, C. *et al*. 2001).

The pORF-CLONE *E. coli/S. cerevisiae* shuttle/expression vector offers an innovative approach for generating cDNA libraries which are significantly enriched for pORFs and express authentic polypeptides. For examle, a randomly primed cDNA library from human fetal brain tissue was cloned in this novel vector, and using robot technology the selected clones were arrayed in microtiter plates and were analyzed by sequencing and for protein expression. In the constructed cDNA expression library, about 60% of clones bear an insert in the correct reading frame. In comparison to unselected libraries it was possible to increase the clones with inserts in the correct reading frame more than fourfold, from 14% to 60%. Using the pORF-CLONE expression system, time-consuming and costly techniques for identification of clones expressing protein by using antibody screening on high-density filters and subsequently rearraying the selected clones in a new “daughter” library can be avoided. The advantage of this vector is that, in a one-step screening procedure, it allows the generation of expression libraries enriched for clones with correct reading frames as sources of recombinant proteins.

3. The pORF-CLONE Vector

The pORF-CLONE vector includes the Cu^{2+}-inducible *CUP1* promoter from the yeast metallothionein gene, which controls expression of genes inserted in the MCS. It also carries the *HIS3* gene, coding imidazol-glycerol-phosphate-dehydrogenase, which enables the selection of ORFs based on histidine prototrophy. In addition, the vector contains the *E. coli* ampicillin resistance (Amp^R) gene for selection in *E. coli* and the yeast selectable markers *URA3* and *LEU2d.* For the improved expression of randomly primed cDNAs, a translation initiation sequence (CAAAATGTCT) that was adapted to *S. cerevisiae* has been introduced allowing the translation of cDNAs without their own start codon. For the detection and purification of expressed gene products, the plasmid includes a tandem epitope tag positioned N-terminal to the multiple cloning site (MCS), consisting of the hemagglutinine epitope (HA) and the oligo histidine domain (RGS-His_6). The modified MCS includes a stop codon in each reading frame and allows the directional cloning (SalI/NotI) of cDNAs. For the cleavage of *HIS3p* from the fusion protein the recognition sequence (6P) of the PreScission protease (CTGGAAGTTCTGTTCCAGGGGCCC) has been additionally inserted. The yeast *HIS3* gene was introduced C-terminal to the MCS (Fig. 2). The translation of the DNA proceeds from the start codon ATG through the tag sequences into the *HIS3* gene. Only if an insert is cloned in the correct reading frame and if it does not contain any stop codon, a complete fusion protein will be produced (Fig. 2B). In this case, expression of the gene product leads to histidine prototrophy in a *HIS3 S. cerevisiaestrain*. Clones bearing the pORF-CLONE plasmid, but without inserts (Fig. 2A), remain auxotrophic, as no *HIS3* gene is expressed because of translation stops in all three reading frames. The yeast cells are also expected to be auxotrophic if they contain a cDNA that is inserted in an incorrect reading frame, leading to internal stop codons and resulting in the termination of translation before reaching the *HIS3* sequence (Fig. 2C, 2D). In general, mRNA sequences carry many stop codons in reading frames other than the correct one.

pORF-CLONE can be used for cloning of cDNAs with SalI/NotI overhangs and for the expression of randomly primed cDNAs.

3.1. Vector Map of pORF-CLONE

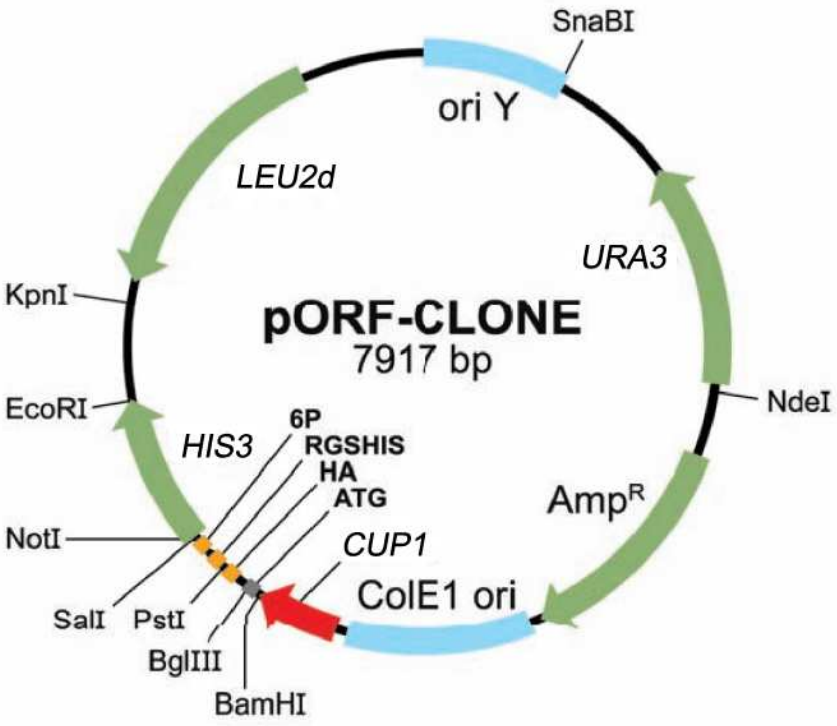

Fig. 1: PlasmidmapofpORF-CLONE.

ColE1 ori: ColE1 origin;
Amp^R: ampicillin resistance;
***CUP1*:** CUP1 promoter;
RGSHis(6): 6xHis tag;
HA: hemagglutinine epitope
6P: PreScission protease recognition sequence
Below: Part of the pORF-CLONE sequence including the cloning sites, tag and protease sequences as well as the translation initiation sequence with the ATG start codon.
The complete DNA sequence is available on request.

BamHI
CACATCAATCATCACATAAAATATTCAGCGAATT*GGATCC*AAA**ATG**TCTGGCAGATCTCA

HA PstI **6xHIS**
C*CCATACGACGTCCC*AGACTACGCT*CTGCAG*AGAGGATCGCATCAC*CATCACCATCAC*

SalI NotI PreScission protease recognition sequence (6P)
GG*GTCGAC*TGAGAGCTCATAGCTAA*GCGGCCGC*CTGGAAGTTCTGTTCCAGGGGCCC

HIS3 ➡
ACAGAGCAGAAAGCCCTAGTAAAGCGTATTACAAATGAAACCAAGATTCAGATTGC

GATCTCTTTAAAGGGTGGTCCCCTAGCGATAGAGCACTCGATCTTCCCAGAAAAAGA

4. Advantages and Applications

1. Efficient, cost-effective selection of cDNA clones with a correct pORF.
2. Background of clones containing cDNA with incorrect reading frames can be reduced more than 10-fold resulting in expression libraries enriched for clones expressing authentic polypeptides.
3. Average insert size of cDNA is approximately 1.2 kb (usage of *E. coli* vectors with *lacZ* as marker gene resulted in insert sizes from 0.1 - 1.0 kb).
4. *S. cerevisiae* host systems allows expression of soluble, correctly folded and modified eukaryotic target proteins in high yields.
5. Improved selection of larger cDNA inserts in the eukaryotic host system - translation problems and frame-shifts can be avoided.
6. Fast generation of high-quality cDNA libraries by means of random priming.
7. Easy, fast and reproducible purification of recombinant proteins via metal chelate affinity chromatography.
8. Detection of recombinant proteins in immunoblotting experiments by commercially available antibodies.
9. Ideally suited for high-throughput cloning or the production of cDNA chips.

5 Kit Components

PORF 10 µg pORF-CLONE vector DNA
500 pmole pORF-1 - PCR Primer
500 pmole pORF-2 - PCR Primer

Shipped at room temperature (RT), store at 4 °C.

6. Protocols

Standard cloning procedures are not described here in detail but can be found in commonly used lab manuals (9, 10).

6.1. Strains, Media and Transformation

E. coli strains XL1-Blue (Stratagene) and DH10B (Life Technology) are used as host strains for cloning and amplification of plasmid DNA. The transformation of recombinant plasmids is performed by electroporation using the Gene Pulser (Bio-Rad) and following the manufacturer's protocol. *E. coli* transformants are selected on LB (0.5% yeast extract, 1% NaCl, 15% bactotryptone) medium supplemented with 100 µg/ml ampicillin. The *S. cerevisiaestrain* GRF18 (α, leu2-3, leu2-2112, his3-15, can1, mal) is used as the eukaryotic expression strain.

pORF-CLONE vector constructs and cDNA expression libraries are transformed in *S. cerevisiae* by the lithium acetate method (Gietz, D. *et al*. 1992), and the resulting transformants are selected on SD-his induction medium (2% dextrose, 0.67% Yeast Nitrogen Base [YNB], 40 mg/l leucine, 0.1 mM $CuSO_4$). *S. cerevisiae* clones can be cultivated in microtiter plates in freezing medium (2% dextrose, 5% glycerol, 0.67% YNB, 40 mg/l histidine, 0.5 M betaine). For protein expression, the clones are transferred in SD-leu medium (2% dextrose, 0.67% YNB, 40 mg/l histidine) and the expression is induced by addition of 0.5 mM $CuSO_4$).

6.2. Sequence Analysis of Transformants

For sequencing, the *S. cerevisiae* clones are transferred into 96-well plates filled with 65 µl freezing medium and incubated 4 days at 28 °C. The insertsof the cDNA clones are amplified by PCR directly from liquid cultures as described previously at Lueking, A. *et al*. 2000. For PCR amplification, the primers pORF-1 (CATATAGAAGTCATCGA) and pORF-2 (TTTGCAG CTACCACATT) are used.

7. References

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8. Order Information, Shipping and Storage

Order#	Product	Quantity
PORF	pORF-CLONE vector DNA	10 µg
shipped at RT; store at 4 °C		