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S. pombe Expression Vector with 6×(His) Tag for Protein Purification and Potential for Ligation-Independent Cloning

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In the area of protein production and purification, researchers have turned to eukaryotic organisms, such as *Schizosaccharomyces pombe*, as hosts for expression of many eukaryotic proteins instead of bacterial organisms. The reasons are many: some bacterial hosts cannot manage eukaryotic genes due to their toxicity, insolubility or their need for posttranslational modifications, such as phosphorylation and acetylation. It is in this area that *S. pombe* is greatly utilized due to its unique traits that closely resemble higher eukaryotic organisms. This includes chromosomal structure and function, cell-cycle control and RNA splicing (15). We have constructed and tested a new plasmid vector, pESP-5 (Figure 1), for gene expression and protein production in *S. pombe*. pESP-5 is derived from pESP-2 (9). This vector contains the *S. pombe* inducible *nmt1* promoter for high-level gene expression (3,11,12) and the 6×(His) affinity tag for protein purification. Similar vectors that contain the 6×(HIS) and GST purification tags and the c-myc, HA and Pk epitope tags have also been constructed for use in fission yeast (2,4). A novel aspect of pESP5 includes the potential of ligation-independent cloning (LIC) (1). The gene of interest can be cloned in the multiple cloning sites by using the conventional restriction fragment ligation method or the LIC method (1,5,7). The LIC strategy allows efficient and directional cloning of the target gene. When specific polymerase chain reaction (PCR) primers are used, the target gene can be placed immediately downstream of the DNA sequences encoding for the epitope tag, FLAG® (Scientific Imaging Systems [Eastman Kodak], New Haven, CT, USA) (6), and sequences for recognition by enterokinase. Because enterokinase cleaves the peptide bond C terminal to its recognition site (8), polypeptides with native amino acid se-

quences can be obtained after removal of the 6x(His) tag by enterokinase.

To prepare pESP-5 for LIC cloning, the vector was linearized with *SphI* and treated with *Pfu* DNA Polymerase (Stratagene, La Jolla, CA, USA) in the presence of 1 mM dTTP. *Pfu* DNA polymerase has both 5' to 3' polymerase activity and 3' to 5' exonuclease activity (10). The 3' to 5' exonuclease activity of *Pfu* DNA polymerase removes nucleotides at the 3' ends of the vector until a dTMP residue is reached, resulting in a vector with 5' extended tails of defined sequence and length at both ends. Because the two tails are noncomplementary to each other, the "LIC-ready" vector ensures directional cloning of the insert with low background, avoiding self-ligation of the vector.

The chicken calmodulin gene was used as a test gene for cloning into pESP-5 using the LIC cloning strategy. Two specifically designed primers with defined "LIC-specific" tails were synthesized and used to PCR-amplify the chicken calmodulin gene. After 25 cycles of amplification with *Pfu* DNA polymerase in a RoboCycler® 40 (Stratagene), the PCR mixture was electrophoresed on a 1% agarose gel, the gel

slice containing the PCR product was excised, and the DNA was recovered using a StrataPrep™ Gel Extraction Kit (Stratagene). The purified PCR fragment was further treated with *Pfu* DNA polymerase in the presence of dATP to generate the LIC-specific tails. *Pfu* DNA polymerase was subsequently removed by extraction with StrataClean® Resin (Stratagene). The LIC-ready pESP-5 vector and the LIC-ready insert DNA fragments can then be annealed at room temperature and directly used for transformation. The length of complementary sequences between the vector and the insert (12 and 13 bases, respectively) are long and stable enough to eliminate the ligation step. The annealed mixture was transformed into *Escherichia coli* cells and selected on LB+amp plates. The cloning efficiency of the calmodulin gene into pESP-5 using LIC strategy was determined by *E. coli* colony PCR, with 92% containing an insertion at the size expected of the calmodulin gene (data not shown).

pESP-5-calmodulin plasmid DNA was then transformed into the *S. pombe* strain SP-Q01. One microgram of the expression construct was mixed with 100 µL of cells (ca. 2×10^8 cells) of the

Figure 1. The pESP-5 vector. Shown is the nucleotide sequence with the corresponding amino acids around the multiple cloning sites. The LIC site and the unique restriction sites in the region are also shown. The stop codon is indicated by an asterisk. Protease recognition sites are indicated on top of the sequences, and the corresponding cleavage sites are indicated with arrows.

host strain SP-Q01 (*leu1-32h⁻*) made competent by using a standard protocol (13). The mixture was incubated at 30°C for 30 min. After the addition of 300 μ L of transformation mixture [40% (wt/vol) polyethylene glycol (PEG) 3350, 0.1 M LiCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA], the cells were further incubated at 30°C for another 30 min. Following a 20-min heat shock at 42°C, the cells were plated on EMM plates (BIO 101, Vista, CA, USA) supplemented with 25 μ M thiamine (repressed conditions) and incubated at 30–32°C for 4–5 days. For storage, single colonies were streaked out on an EMM+25 μ M thiamine plate, and the strains were stored at -70°C as a cell suspension in 20% glycerol.

For protein induction, the expression strain was inoculated into 5 mL yeast extract plus supplements (YES) media and incubated at 30°C overnight. A portion of the overnight culture was used to inoculate another 10 mL YES (optical density [OD]₆₀₀ = 0.1). After 4–5 h of further incubation at 30°C for the culture to reach mid-log phase (OD₆₀₀ = 0.5), the cells were collected by centrifugation at 1000 $\times$ *g* in a benchtop centrifuge and washed once with 50 mL sterilized water. The cells were then resuspended into 10 mL of EMM media with 25 μ M thiamine (for

repressed condition) or without thiamine (for induced condition) and grown at 30°C for 18–20 h. The cells were collected and washed once with lysis buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 10 mM imidazole), and the cell pellet was further resuspended in 500 μ L lysis buffer. Five grams of acid-washed glass beads (0.4–0.6 mm in diameter; Sigma, St. Louis, MO, USA) were added, and the cells were broken by vortex mixing at 4°C for 5 min. After centrifugation in a microcentrifuge for 5 min at 12000 $\times$ *g*, the supernatant (crude lysate) was saved and stored at -70°C for further purification and analysis.

For purification of 6 $\times$ (His) fusion proteins, cleared lysate was subjected to a one-step purification using the Ni-NTA resin (14) provided in the QIAexpress™ Ni-NTA Spin Kit (Qiagen, Valencia, CA, USA). Briefly, 200 μ L of cleared lysate were loaded onto a pre-equilibrated Ni-NTA spin column. After centrifugation at 700 $\times$ *g*, the column was washed three times with the wash buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl) containing 20 and 40 mM imidazole, respectively. The fusion protein was then eluted with 50 μ L elution buffer (50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl, 250 mM imidazole) twice and combined. Twenty micrograms of

Figure 2. Expression and purification of chicken calmodulin protein. A chicken calmodulin gene was inserted at the LIC site of pESP-5 vector and expressed in *S. pombe*. (A) Shown is a Coomassie Blue stained 4%–20% Tris-glycine-sodium dodecyl sulfate (SDS) gel (Novex) with samples derived from various stages of the purification procedure. Lane 1 is crude lysate; lane 2 is a flow-through sample of the lysate after subjection to the one-step purification for 6 $\times$ (His) fusion proteins, using the Ni-NTA resin; lanes 3, 4 and 5 are washes with 20, 20 and 40 mM imidazole, respectively; lanes 5 and 6 are products eluted with 250 mM imidazole. The lane containing marker proteins (Novex) is indicated. (B) Shown is a Coomassie Blue stained 4%–20% Tris-glycine-SDS gel with 6 $\times$ (His)-calmodulin before and after enterokinase digestion. Lane 1, 6 $\times$ (His)-FLAG-calmodulin; lanes 2, 3 and 4 are samples of the fusion protein incubated with enterokinase for 2, 4 and 10 h, respectively. The cleavage reaction was carried out in a vol of 80 μ L with 20 μ g of 6 $\times$ (His)-calmodulin fusion protein and 1 U of enterokinase at 37°C.

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purified HIS6-calmodulin fusion protein were incubated with 1 U of enterokinase (Stratagene) at room temperature for 0, 2, 4 and 10 h, respectively. A fraction of the sample was electrophoresed on a 4%–20% Tris-glycine-acrylamide gel (Novex, San Diego, CA, USA) and stained with Coomassie® Blue. Figure 2A shows the expression and purification of 6×(His)-calmodulin fusion protein. The level of induced expression of 6×(His)-calmodulin is estimated to be 5%–10% of the total soluble protein. Note the extra bands appearing on the gel are probably due to the endogenous His proteins contained within the cell that are co-purified with the target. As shown in Figure 2B, calmodulin protein without any extraneously added amino acid residues was obtained by removing the 6×(His) purification tag with enterokinase. Before enterokinase cleavage, the fusion protein was dialyzed against the enterokinase cleavage buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM CaCl₂) using Amicon® Microcon®

Microconcentrators (Millipore, Bedford, MA, USA). The FLAG epitope can be tagged to the protein of interest and used in immunoprecipitation experiments. The 6×(His)-FLAG-calmodulin fusion protein was detected through a western blot using the M2 anti-FLAG antibody (Scientific Imaging [Eastman Kodak]) (data not shown).

In conclusion, the new pESP-5 *S. pombe* expression vector allows efficient and directional cloning of PCR products immediately downstream of the enterokinase recognition/cleavage site. Thus, polypeptides without extraneously added amino acid residues can be obtained by removing the purification tag with enterokinase after purification of the recombinant 6×(His) fusion protein.

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Black Cellular Spreading and Motility Assay

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Here, we describe a new technique to study cell spreading and motility on black ink particle-coated substrates. The experimental technique is simple and is based on the observation that cells migrating on a glass substrate, which is densely and evenly coated