The amplification from the crude lysates of the recombinant *P. pastoris* strains KM71 (pPIC9-MYO)-Mut³ and GS115 (pPIC9-MYO)-Mut⁴, which contained the integration of the myostatin gene to chromosomal DNA, was completed. When Taq DNA polymerase was used for amplification, we observed either no or nonspecific amplification products. The positive control sample yielded the 831-bp specific fragment. When the HOTStar Taq DNA polymerase was used for the amplification, the 831-bp specific amplification product was visible. The wild-type gene AOX1 2.2-kb fragment was co-amplified in GS115-derived strains. The fragment is not visible in KM71-derived strains, because the gene is deleted from the parent strain.

The highest yield was attained with 10 min of boiling at 80°C (Figure 1). Changing the volume of amplification mixture did not influence the result. No changes were found when the annealing temperature and number of amplification cycles were changed.

Similar results were observed when the recombinant yeast *P. pastoris* strain KM71 (pPIC9-LEP)-Mut⁵ with cloned gene for leptin was amplified using the same procedure. The results are demonstrated in Figure 2. In this experiment, a specific 912-bp product was observed as a positive result. No product from the 2.2-kb wild-type gene fragment was visible in these KM71-derived strains.

From the presented results we can deduce that crude yeast lysates contain many PCR inhibitors, which decreases the specificity of the amplification and the amount of amplification product. In contrast to the amplification from the crude lysates of some bacterial cells, only some robust Taq DNA polymerases can overcome these inhibitors. However, fundamentally, the amplification from crude yeast lysates is possible, and the described method can be used as an inexpensive and quick alternative to isolation of chromosomal DNA from yeast cells.

**REFERENCES**


**Mammalian Expression Vectors for Epitope Tag Fusion Proteins that Are Toxic in *E. coli***

Expression of fusion proteins with enhanced GFP (EGFP) or peptide epitope tags such as Myc, HA, or FLAG is a common tool to study the function or location of proteins in mammalian cells. EGFP is an optimized variant of the GFP of the jellyfish *Aequorea victoria* (2,5,7,14). EGFP fusion proteins can be detected by fluorescence microscopy of living or fixed cells or by Western blot with EGFP antibodies. The Myc epitope tag consists of amino acids EQKLISEEDL within the C-terminal domain of human c-Myc (3,11). The HA epitope tag consists of amino acids YPYDVPDYA of the influenza hemagglutinin (4,11). The FLAG® epitope (Sigma-Aldrich, St. Louis, MO, USA) consists of the eight amino acid residues DYKDDDDK (6). Recombinant proteins containing the Myc, HA, or FLAG epitope can be detected by immunofluorescence or by Western blot using antibodies against the Myc, HA, or FLAG epitope. In addition, EGFP-, Myc-, HA-, or FLAG-tagged recombinant proteins can be specifically immunoprecipitated from cell lysates with antibodies against the EGFP, Myc, HA, or FLAG tag. Previously, we have reported pKoz/M-FLAG and pKoz/EGFP cloning vectors for the generation of FLAG- or EGFP-tagged cDNA constructs (10). The pKoz vectors are suitable for cloning of genes that are toxic in *E. coli* because prokaryotic transcription termination signals prevent nonspecific basal expression in *E. coli*. However, the pKoz vectors are not suitable for direct expression in mammalian cells, and FLAG- or EGFP-tagged cDNA constructs need to be subcloned into a mammalian expression vector. Here we report the generation of pExpress vectors for the transient expression of EGFP, Myc, HA, or FLAG fusion proteins in mammalian cells. The pExpress vectors (Figure 1) contain the same vector backbone as the pKoz vectors including a β-lacta-
mase (bla) gene for ampicillin resistance, a Col E1 origin, and three sets of the rrnB terminators T₁ and T₂ to suppress readthrough transcription from cryptic start sites on the plasmid (1). However, the pExpress vectors have a cytomegalovirus (CMV) immediate early promoter inserted 5′ of the epitope tag, which drives expression of EGFP, Myc, HA, or FLAG fusion proteins.

To create pExpress/EGFP (Figure 1), the CMV promoter (610 bp) was amplified by PCR from the pEGFP-N3 vector (BD Biosciences Clontech, Palo Alto, CA, USA) and inserted between the HindIII and SalI sites of pKoz/EGFP. The stuffer insert was excised by digest with NdeI and BamHI and replaced by a 44-bp synthetic double-stranded oligonucleotide with flanking NdeI and BamHI sites and an internal KpnI site. The resulting pExpress/EGFP vector has a 610-bp CMV promoter including an enhancer region and a TATA box, a Kozak sequence for ribosome binding (12), and the EGFP open reading frame. cDNAs can be inserted by in-frame ligation between the 5′ cloning sites NdeI or KpnI and the 3′ cloning sites BamHI, XbaI, or NotI. To create pExpress/Myc, pExpress/HA, or pExpress/FLAG, the Koz/EGFP sequence was excised by digest with PacI and NdeI and replaced with a Koz/Myc, Koz/HA, or Koz/FLAG sequence. The Myc, HA, and FLAG epitope tags contain a start codon encoding a methionine 5′ of the epitope sequence. The cloning sites for insertion of cDNAs are identical to pExpress/EGFP. In-frame ligation of cDNA inserts into pExpress/EGFP, pExpress/Myc, pExpress/HA, and pExpress/FLAG can be used to express recombinant protein with an N-terminal EGFP, Myc, HA, or FLAG tag in mammalian cells.

To test the pExpress vectors, we subcloned p21-activated protein kinase 2 (PAK-2) (8,9) between the NdeI and BamHI sites of pExpress/EGFP and pExpress/FLAG to obtain pExpress/EGFP-PAK-2 and pExpress/FLAG-PAK-2; the regulatory domain (RD) of PAK-2 was subcloned between the NdeI and XbaI sites of pExpress/Myc to obtain pExpress/Myc-RD; the Arp2 subunit of the Arp2/3 actin polymerization complex (13,15) was subcloned between the NdeI and NotI sites of pExpress/HA to obtain pExpress/HA-Arp2. The plasmids pExpress/EGFP-PAK-2, pExpress/FLAG-PAK-2, pExpress/Myc-RD, and pExpress/HA-Arp2 were transfected into human embryonic kidney 293T cells by lipofection with GeneJammer® (Stratagene, La Jolla, CA, USA), and cells were grown for 24–48 h. Expression of EGFP-PAK-2 was monitored by fluorescence microscopy (Figure 2A). Recombinant EGFP-PAK-2 was detected in the cytoplasm of transfected cells. Cells transfected with pExpress/FLAG-PAK-2, pExpress/Myc-RD, and pExpress/HA-Arp2 were lysed and analyzed by Western blot with monoclonal anti-Myc (Invitrogen, Carlsbad, CA, USA), monoclonal anti-HA (Covance, Berkeley, CA, USA), and monoclonal anti-FLAG M2 (Sigma-Aldrich) antibodies (Figure 2B). Recombinant Myc-
Benchmarks

Figure 2. Expression of EGFP-, Myc-, HA-, and FLAG-tagged fusion proteins. cDNAs for PK-2, the regulatory domain (RD) of PK-2, and the Arp2 subunit of the Arp2/3 actin polymerization complex were subcloned into pExpress vectors to generate pExpress/EGFP-PK-2, pExpress/Myc-RD, pExpress/HA-Arp2, and pExpress/FLAG-PK-2. Plasmids were transfected into 293T cells, and expression of recombinant protein was analyzed at 24–48 h after transfection. (A) Expression of EGFP-PK-2 was detected by fluorescence microscopy. (B) Expression of Myc-RD, HA-Arp2, and FLAG-PK-2 was detected by Western blots with antibodies against Myc, HA, and FLAG epitopes. Positions of molecular weight (MW) markers (Pierce Chemical, Rockford, IL, USA) are indicated at the left.

The pExpress vectors described here can be used to transiently express EGFP-, Myc-, HA-, or FLAG-tagged recombinant protein in mammalian cells. Tagged recombinant protein can be detected by Western blot with antibodies against EGFP, Myc, HA, or FLAG. In the case of pExpress/EGFP, fusion proteins can be detected in live cells by fluorescence microscopy. This allows the determination of the subcellular localization of EGFP fusion proteins and the tracking of changes of subcellular localization in response to specific treatments. Myc-, HA-, or FLAG-tagged recombinant proteins can be used for co-localization studies using immunofluorescence or protein-protein interaction studies using co-immunoprecipitation. The pExpress vectors are a further development of the previously reported pKoz vectors. Similar to the pKoz vectors, they are suitable for cloning of genes that are highly toxic in E. coli, especially after in vitro procedures such as PCR or site-directed mutagenesis. For example, cloning of in vitro polymerized PK-2 into vectors without prokaryotic transcription termination signals results in low numbers of clones and a high percentage of false clones with spontaneous mutations, because mutations that abrogate protein kinase activity are not toxic and therefore have a growth advantage. Cloning into pKoz or pExpress vectors increases the number of total clones and greatly reduces the percentage of false clones with spontaneous mutations. However, unlike pKoz vectors, pExpress vectors can be used directly with no need for further subcloning for transient expression of inserted genes in mammalian cells.

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