and cycling conditions can be used in the single-tube nested PCR devised. The first round of amplification includes limiting the concentrations of primers so that they are exhausted at the completion of the first PCR. Since the concentration of the outer primers is only 0.1 μ M, the larger product synthesized by them does not contribute significantly to the overall yield of products; the larger product serves only to boost the second stage amplification reaction. When the first PCR was performed, the primers for the second PCR were not available to anneal to the template. When the second PCR was running, the outer primers were not practically available because they were almost completely used up during the first amplification.

In schistosomiasis, several applications for PCR can be envisaged: detection of parasites in infected water collections, as well as diagnosis of infection in vertebrate and invertebrate hosts. Indeed, a few approaches based on the detection of *Schistosoma* DNA were published for the detection of the parasite in snails (3,6) and cercaria contaminated water collections (2). The diagnostic system proposed here can be easily adapted to be used for PCR detection of other infectious pathogens. In this direction, we have also adapted the single-tube nested PCR for the diagnosis of malaria (unpublished results).

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PCR-Based Method for Identification of Integration Events in the *Pichia pastoris* Genome

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The yeast *Pichia pastoris* is a frequently used microorganism for the cloning and expression of eukaryotic genes. These yeast cells have many of the advantages of higher eukaryotic expression systems, such as protein processing, protein folding, and posttranslational modification. *P. pastoris* is a methylotrophic yeast that is capable of metabolizing methanol as its sole carbon source. The promoter regulating the production of alcohol oxidase is used to drive heterologous protein expression in yeast. Stability of recombinant molecules is ensured by homologous recombination between transforming DNA and regions of homology within the genome. Such integrants show extreme stability in the absence of selective pressure.

The presence of a recombinant gene in the appropriate site of the yeast genome is usually confirmed by PCR on isolated genomic DNA. Isolation of genomic DNA is a laborious and time-consuming method, which moreover requests expensive lytic enzymes for the digestion of yeast cell walls. Alternatively, glass-bead-based extraction methods are used for the rapid extraction of DNA for PCR. For routine applications in our laboratory, we have developed a method that is based on the

Benchmarks

amplification of a crude lysate of yeast cells instead of the isolated yeast chromosomal DNA. Routinely, non-purified DNA released from boiled cells is sufficient for PCR, although there are a lot of PCR inhibitors in such crude lysates that can negatively influence the function of *Taq* DNA polymerase. No system for amplification from *P. pastoris* crude lysates has been described so far.

E. coli DH5 α TM strain was obtained from BD Biosciences Clontech (Palo Alto, CA, USA). *P. pastoris* strains KM71 and GS115, as well as the parent plasmid pPIC9, were obtained from Invitrogen (The Netherlands) and were described in detail previously (1).

Recombinant *P. pastoris* strains KM71 (pPIC9-MYO)-Mut^S and GS115 (pPIC9-MYO)-Mut⁺ with the cloned part of human myostatin gene (GenBank[®] accession no. AF019627) (2) and recombinant *P. pastoris* strain KM71 (pPIC9-LEP)-Mut^S with cloned human *ob* gene (accession no. U18915) (3) were used for confirmation of integration of the genes to chromosomal DNA.

Media components were from Sigma (St. Louis, MO, USA), and media and cultivation conditions were described previously (1). Single colonies of individual strains were cultivated on a minimal medium with glycerol for 48 h at 30°C.

Two commercially available polymerases [*Taq* DNA polymerase (Sigma) and HOTStar *Taq* DNA polymerase (Qiagen, Hilden, Germany)] were used for PCR.

The crude cell lysates were prepared by resuspension of a single yeast colony taken from medium in 400 μ L distilled water in a screw-cap microcentrifuge tube. The suspension was centrifuged at 14 000 $\times$ *g* for 5 min, washed twice with distilled water, and resuspended in 400 μ L distilled water again. This suspension was then heated at 80°C and 100°C for 5, 10, or 15 min before being centrifuged for 5 min at 14 000 $\times$ *g* to pellet the cell debris. A total of 1 μ L of the supernatant, containing genomic DNA, was used for subsequent PCR amplification.

The primers 5'AOX1 (5'-GACTG-GTTCCAATTGACAAGC-3') and 3'AOX1 (5'-GCAAATGGCATTCTG-ACATCC-3') are based on plasmid pPIC9 sequences adjacent to a cloning site and enable the amplification of a 831-bp specific fragment in the case of insertion of the gene for myostatin (pPIC9-MYO constructs) and 912-bp fragment in the case of *ob* insertion (pPIC9-LEP constructs). The length of PCR product from an empty plasmid pPIC9 was 492 bp. The expression plasmid pPIC9, the recombinant plasmid

pPIC9-MYO, and the *E. coli* (pPIC9-MYO) crude cell lysate were used as positive PCR controls. PCR was carried out in final volume of 50 μ L containing 1 μ L DNA supernatant containing approximately 10 ng genomic DNA, 10 pmol each primer, and 1 U *Taq* DNA polymerase. DNA samples were first completely denaturated by incubation at 94°C for 5 min. DNA was amplified by subjected to 35 cycles of denaturation at 95°C for 35 s, primer annealing at 50°C for 30 s, and elongation at 72°C for 90 s, using a model PTC-150HB thermal cycler (MJ Research, Waltham, MA, USA). After the last amplification cycle, the samples were incubated further at 72°C for 5 min for complete elongation of the final PCR products. After the PCR, the amplification results were visualized by performing 1% agarose gel electrophoresis (5 V/cm; 30–45 min) in 1 $\times$ TBE buffer and ethidium bromide staining.

First, we have optimized our amplification conditions on a crude lysate of the bacteria *E. coli*. The conditions described earlier were found to be optimal for the amplification. The bacterial cells *E. coli* contained the same recombinant plasmids that were used to transform the yeast *P. pastoris*. The *E. coli* lysates were then used as a positive amplification controls.

Figure 1. An example of amplification from myostatin integrants. Lanes 1 and 12, 100-bp ladder. Lane 2, plasmid pPIC9; lane 3, plasmid pPIC9-MYO; lane 4, crude lysate from *E. coli* (pPIC9-MYO); lane 5, GS115 (pPIC9-MYO)-Mut⁺ culture, not treated; lanes 6–8, GS115 (pPIC9-MYO)-Mut⁺ lysate heated at 80°C for 5, 10, and 15 min, respectively; lanes 9–11, GS115 (pPIC9-MYO)-Mut⁺ lysate heated at 100°C for 5, 10, and 15 min, respectively.

Figure 2. An example of amplification from leptin integrants. Lanes 1 and 10, 100-bp ladder; lane 2, plasmid pPIC9; lane 3, KM71 (pPIC9-LEP)-Mut^S culture, not treated; lanes 4–6, KM71 (pPIC9-LEP)-Mut^S lysate heated at 80°C for 5, 10, and 15 min, respectively; lanes 7–9, KM71 (pPIC9-LEP)-Mut^S lysate heated at 100°C for 5, 10, and 15 min, respectively.

Benchmarks

The amplification from the crude lysates of the recombinant *P. pastoris* strains KM71 (pPIC9-MYO)-Mut^S and GS115 (pPIC9-MYO)-Mut⁺, 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)-Mut^S 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.

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

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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-