Antibody Screening for Secreted Proteins Expressed in *Pichia pastoris*


The methyl-trophic yeast *Pichia pastoris* is becoming widely used as an organism for high-level protein expression (3). Commercially available vectors and host strains allow for secreted or intracellular expression of heterologous protein. When the desired end result is secretion, it is useful to have a quick and simple screening assay for secreted protein. We describe a semi-quantitative colony blotting protocol for the detection of proteins secreted by *P. pastoris* growing on agar plates.

Vectors using the HIS4 gene to allow growth on media without histidine, and using the alcohol oxidase (AOX1) control sequences to allow induction by methanol, are commonly used for expression of proteins in *P. pastoris* (3,7,8). Screening generally takes the form of selection on plates lacking histidine to ensure that the HIS4 gene is integrated, followed by comparative growth on minimal dextrose or methanol plates to determine the methanol utilization (Mut) phenotype. Mut* colonies grow fast on methanol plates because the natural AOX1 gene is undamaged, whereas Mut* colonies grow slowly because the transfected gene has undergone homologous recombination into the AOX1 gene. Screening for integration of the gene by polymerase chain reaction (PCR) or dot blotting may also be desirable. The latter is particularly important in screening for multiple integrations, which can increase protein expression (2).

Screening of colonies for secreted product is performed by growing cells in minimal glycerol medium, then concentrating them and continuing culture in minimal methanol medium. This is followed by gel electrophoresis and frequently by Western blotting. Screening a large number of colonies in this way is time-consuming and requires substantial effort. The protocol described below requires very little hands-on time and allows screening for high-expressor secreting colonies to "piggyback" on the Mut*/*Mut* comparative screening, thus bypassing screening for high-copy-number integrants by DNA dot blotting.

After transfection and selection for incorporation of the DNA on regeneration dextrose base (RDB) agar plates and/or G418 plates [i.e., for His+ phenotype and for multicopy integration, respectively (6)], colonies are picked and touched onto minimal methanol (MM) and minimal dextrose (MD) plates sequentially. After incubation at 30°C for 1–2 days, large colonies are present on the MD plates. The MM plates have either small (Mut*) or large (Mut*) colonies. At this point, the colonies are scored for the Mut phenotype, and the MD plates are stored at 4°C. A nitrocellulose filter (Schleicher & Schuell, Keene, NH, USA) is placed over the colonies on the MM plates, followed by three sheets of 3MM chromatography paper (Whatman, Maidstone, Kent, England, UK). Several towels are added on top, and the pile is weighted down with about 50 g. The plate is incubated at 30°C for 1–3 h. Omitting the blotting step, simply laying the nitrocellulose filter on top of the colonies is less sensitive, which can be an advantage in selecting high-secreting colonies (Figure 2). The nitrocellulose filter is removed and washed in tris-buffered saline (TBS: 20 mM Tris base, 137 mM NaCl, pH 7.6) to remove the yeast colonies. The next steps are identical to standard Western blotting protocols and require specific antibody reagents for the particular proteins under investigation (see below). High-expressing colonies identified in this manner are recovered from the MD plate and grown up for more specific assays of protein expression.

In the example presented here, we transfected *P. pastoris* strain GS115 with a gene for the T cell receptor (TCR) α-chain cloned into pPIC9 (6) (Invitrogen, San Diego, CA, USA). The TCR α-chain gene was truncated to allow secretion (4,5). The cells were transfected by electroporation and plated onto RDB plates to select for incorporation of the HIS4 gene. We tested colonies growing on RDB for incorporation of the transfected gene by standard techniques; MM vs. MD plates for Mut phenotype, followed by DNA dot blotting for α-chain incorporation. For this demonstration experi-
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colonies of various genotypes were picked onto an MM plate, grown and blotted as described above. Picking colonies from a previously grown MD plate (as recommended by Invitrogen for more accurate Mut phenotype determination) gives more uniformly sized colonies, making comparison of relative secretion levels easier. The filters were blocked with 10% nonfat dry milk in TBS with 0.1% Tween® 20 (TTBS), washed and probed with antibodies. The hamster IgG Mab H28-710 recognizes a determinant on the mouse TCR α-chain constant domain (1). The filters were incubated sequentially with H28-710 (1 µg/mL in TTBS), goat anti-hamster IgG-biotin (0.5 µg/mL) (Caltag, South San Francisco, CA, USA), and streptavidin-horseradish peroxidase (1/4000) (Vector Laboratories, Burlingame, CA, USA). Three washes in TTBS were performed between incubations. The filters were developed with an enhanced chemiluminescence (ECL™) kit (Amersham, Little Chalfont, Bucks, England, UK). The results are shown in Figure 1. Most, but not all, of the colonies that had incorporated α-chain DNA were found to secrete α-chain protein at varying degrees. In addition, 6/7 colonies with unknown genotype (picked from the transfection plate) secreted α-chain protein. Several of the colonies (Nos. 3, 6, 12 and 17) secreting α-chain proteins were tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, confirming that α-chain protein was indeed secreted. Colony No. 17 secreted significantly less α-chain than the other three colonies (not shown).

To demonstrate that this method could distinguish between levels of secretion, to screen for high-secreting colonies, we grew up colony No. 12 from Figure 1 and a negative control human serum albumin (HSA)-secreting colony to 0.5 OD. The cells were mixed at various ratios, and the mixed cultures

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**Figure 1.** Secretion of TCR α-chain protein detected by colony blotting. (a) A grid of P. pastoris colonies transfected with TCR α-chain in pPIC9 was grown on an MM plate. (b) A key to the grid. The labels α+ or α− refer to the genotype of the colony previously determined by DNA dot blotting (2). Colonies Nos. 12–18 have not been tested by DNA dot blot and are therefore labeled “?”. Control colonies secreting HSA (colonies Nos. 8 and 23) or expressing intracellular β-galactosidase (No. 10) showed negligible background in this assay. Grid positions 7, 9 and 11 had no colony. Most of the colonies that had been shown to have integrated α-chain DNA secreted α-chain detectable in this assay. Two colonies, Nos. 24 and 25, did not secrete α-chain protein even though they had incorporated >4 copies of α-chain DNA. Previously untested colonies Nos. 12–14 and Nos. 16–18 were shown by this assay to secrete α-chain protein.

**Figure 2.** Distinguishing different levels of secretion. TCR α-chain expressing colony No. 12 and an HSA-secreting colony were grown up and mixed at different ratios. The mixed colonies were grown on MM plates and screened. The mixed colonies grew as well as each other on both MM and MD plates (not shown). (a) The plate was blotted as for Figure 1. (b) The plate was overlaid with nitrocellulose filter, but not blotted. (c) Key showing the percentage of colony No. 12 in each mixed colony, the remainder being the HSA secretor.

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were picked onto MM plates and grown as before for 24 h. Replica plates were overlaid with nitrocellulose. One filter was blotted as described above (Figure 2a) and for the other, the nitrocellulose was removed after 10 min (Figure 2b). There is a clear titration in the strength of the ECL signal with the dilution of the TCR α-chain expressing cells. Without blotting, the signal was indistinguishable from background when the α-chain expressing cells represented 12.5% of the culture (Figure 2b). With blotting, the signal was clear at this dilution and was still distinguishable from background with 6.25% α-chain expressing cells. Thus, the technique can be used for screen for colonies expressing both α- and β-chains of the TCR.

We have successfully used this method to screen for colonies expressing both β- and α-chains of the TCR.

REFERENCES


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Detection of Early and Late Stage Apoptosis with Field Inversion Gel Electrophoresis


The detection of apoptosis by gel electrophoresis initially involved the use of static gel electrophoresis for assessing DNA fragmentation to nucleosomes and nucleosome multimers (4). This approach is based on the assumption that DNA “ladder” formation is the “hallmark” of apoptosis and neglects the relevance of intermediate formation of 50 and 300 kb DNA, which in some cases may represent the only DNA fragmentation associated with apoptosis (2,3,9).

We describe here a procedure for visualizing both large (50 and 300 kb) and small (nucleosome multimers) DNA fragments formed during apoptosis. This procedure is not intended to replace either the conventional pulsed-field gel electrophoresis (PFGE) or static gel electrophoresis, but to provide a means of assessing the nature of apoptotic chromatin fragmentation before other procedures are applied. When analyzing large DNA fragments, it is important to eliminate any mechanical or chemical breakage of the DNA since this will markedly alter the resulting patterns. This can be accomplished by embedding whole cells into small agarose blocks, which are incubated in a digestion buffer, containing detergent and proteinase K, for 24–48 h. This procedure results in cell lysis and exposes genomic DNA and larger DNA fragments, which can be resolved by PFGE. The smaller DNA fragments (nucleosomes and nucleosome multimers) tend to diffuse out of the agarose blocks during the digestion procedure. This will result in the loss of the DNA “ladder” pattern typical of late-stage apoptosis. These small fragments can be recovered by extracting the DNA, which diffuses into the digestion buffer, and then using static gel electrophoresis (12), but recoveries can be low, and some DNA will likely be lost. A new method for detection of the large DNA fragments, formed during the initial stages of apoptosis, was described previously (12). Since these migrate only a short distance into a 1% agarose gel during conventional electrophoresis, it has been suggested that static gel electrophoresis can be used to detect the presence of 50-kbp DNA, with PFGE then used to obtain a better delineation. Sodium dodecyl sulfate (SDS) was used to deproteinize the DNA, with the resulting lysate loaded directly into gel wells.

We have found that classical PFGE procedure, especially the lengthy proteinase K digestion, can result in nonspecific DNA fragmentation even when DNase-free proteinase K is used. The use of SDS to deproteinize the cells circumvents this problem, but naked DNA liberated by SDS deproteinization is very vulnerable to mechanical force. Even gentle pipetting of the naked DNA will result in some nonspecific fragmentation. In our hands, PFGE can be used to separate 50 kb DNA from nucleosome oligomers with the following modifications of the original