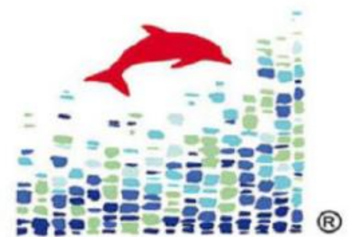
pPICHOLI Shuttle Vector System

Order #
PPICH



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1. Summary

The pPICHOLI vectors have been designed for heterologous gene expression in the yeast *Pichia pastoris* as well as in the prokaryote *Escherichia coli*. The vectors contain an inducible (yeast) alcohol oxidase (AOX) promoter (*except for: pPICHOLI-C = CUP1 promoter*) and an *E. coli* T7 promoter as well as sequences allowing autonomous replication both in *P. pastoris* and *E. coli* (pPICHOLI-C has no T7 promoter). Thus, vector linearization is no longer required and small amounts of DNA are sufficient to successfully transform *P. pastoris*. The integrated PARS sequence enables simple recovery of plasmids from yeast. Time-consuming subcloning into a number of expression vectors, including testing for a successful gene expression, is no longer necessary. A multiple cloning site (MCS) enables convenient ligation of DNA fragments into the vectors.

The dual expression system offers many advantages: the prokaryotic expression system is simple to handle and allows a cost-effective and high-level production of heterologous proteins. The *P. pastoris*/pPICHOLI system is a powerful eukaryotic expression system showing rapid growth at high densities combined with the strong AOX or CUP1 promoter, respectively. It is ideally suited for expression of soluble proteins with post-translational modifications and those (eukaryotic) proteins causing problems when expressed in *E. coli* (e.g., proteins toxic to *E. coli*).

pPICHOLI-C carries, instead of the AOX promoter, the copper-inducible CUP1 promoter of *Saccharomyces cerevisiae* which has been shown to reduce the induction time greatly. It has no T7 promoter and is not suited for expression in *E. coli*.

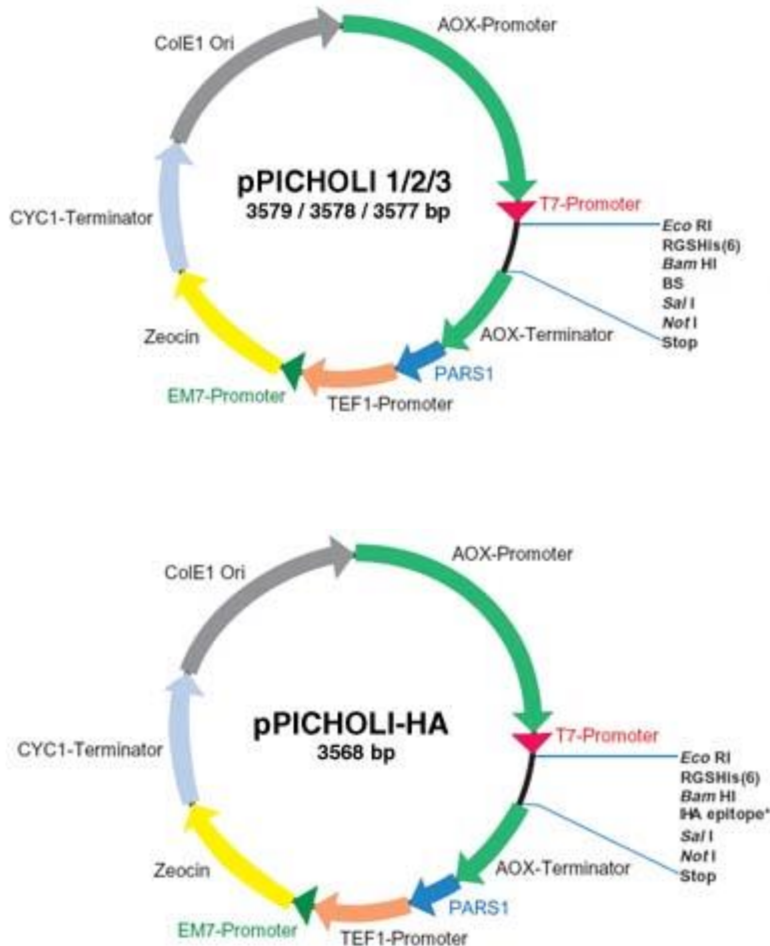
2. Introduction

The well-studied bacterium *E. coli* is a commonly used organism and a pioneer host in heterologous gene expression and production of recombinant proteins. This prokaryotic expression system is simple to handle, cost-effective, and produces large amounts of target proteins (1-3). However, despite these advantages it sometimes shows problems when expressing certain genes, especially eukaryotic genes. As a result of, for instance, differences in the prokaryotic and eukaryotic codon usage this often leads to the production of aggregated and denatured proteins, accumulated in inclusion bodies, and only a small fraction matures into the desired native form. Alternatively, eukaryotic expression systems have been developed to obtain more soluble protein, which, in addition, may undergo some eukaryotic post-translational modifications. The soluble proteins are better sources for crystallization and NMR studies since they are more likely to show a functional folding. They are also more suitable for the generation of native protein chips in high density. Yeast expression systems, including the methylotrophic yeast *P. pastoris*, have been used over the last years as powerful expression systems for a number of heterologous genes (4-7). However, both eukaryotic and prokaryotic systems have their advantages and disadvantages. Therefore, choosing a suitable expression system for a particular protein is always critical and a compromise, depending primarily on the properties of the protein, the amounts required, and its intended purpose. In the past, heterologous gene expression often was tested empirically, and a number of host organisms had to be tested. Taking this into account, an expression vector allowing protein expression in both prokaryotic and eukaryotic systems greatly reduces workload, time, and costs involved.
MoBiTec's pPICHOLI vectors avoid labor-intensive and costly subcloning procedures. They have been constructed for inducible protein expression in the two commonly used hosts *E. coli* and *P. pastoris* (8). Similar to *E. coli*, *P. pastoris* is known for its ability for rapid growth at high cell density and when combined with a strong promoter, has, in a number of cases, yielded up to several grams of the heterologous protein per liter of culture (4, 9). In *P. pastoris* 100% of the cloned genes could be expressed as soluble proteins, whereas in *E. coli* only 86% of the cloned genes were successfully expressed (27% of them as soluble proteins).

3. The pPICHOLI Vectors

The dual expression vectors pPICHOLI and pPICHOLI-HA combine eukaryotic and prokaryotic promoter elements. Phage T7 promoter, including the ribosomal binding site of the major capsid protein, promotes the efficient bacterial expression and is placed downstream from the *P. pastoris* promoter. The strong alcohol oxidase promoter (AOX) is tightly regulated, that is, protein expression is completely repressed when grown on glucose and maximally induced when grown on methanol (10).
pPICHOLI is available with a multiple cloning site in three different reading frames to simplify cloning in frame with the tags (pPICHOLI-1, pPICHOLI- 2, pPICHOLI-3). pICHOLI-1 (3579 bp) has two G bases directly upstream of the SalI site. pPICHOLI-2 (3578) and pPICHOLI-3 (3577 bp), respectively, are lacking one or both of these G bases. pPICHOLI-C carries the CUP1 promoter of *Saccharomyces cerevisiae* (instead of the AOX promoter) which has been shown to reduce the induction time greatly (11, 12).
pPICHOLI-C has no T7 promoter and is not suited for prokaryotic protein expression. Due to the use of a common selection marker zeocin, the sizes of the shuttle vectors remain small (~3.6 and 3.2 kb, respectively), hence they remain convenient for handling, cloning, and transformation. By integration of a *Pichia*-specific autonomous replicating sequence (PARS1) into the pPICHOLI vectors, linearization is no longer required, and the transformation efficiency is increased to about 10^5 transformants/µg DNA (6). Additionally, plasmids can be easily recovered from *P. pastoris*. The pPICHOLI dual expression vectors include a double tag consisting of an RGS(His)6 and an *in vivo* biotinylation sequence (13) for sensitive detection and rapid purification of expressed proteins. Owing to the strong affinity of biotin to avidin, capture and screening assays are thus greatly facilitated. pPICHOLI-HA (3568 bp) lacks the biotin-tag, but instead possesses an HA (hemagglutinin) epitope.

3.1. Vector maps of pPICHOLI and pPICHOLI-C



pPICHIA-1/-2/-3 and -HA: ColE1 ori: ColE1 origin of replication; AOX promoter: alcohol oxidase promoter; Zeocin: zeocin resistance; PARS1: *P. pastoris* autonomous replicating sequence; RGSHis(6): Poly(His)-Tag; BS: biotinylation sequence;

```
             EcoR I                                        RGSHis(6)
CAATTTCACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCGCATCACCAT

           BamH I     Biotinylation Sequence (BS)
CACCATCACGGATCCCTGAACGACATCTTCGAAGCTCAGAACATCGAATGGAAAAAGG

 Sal I               Not I
GTCGACCTGCAAGATCTGCGGCCGCGAATTAATTCGCCTTAGACATGACTGTTCCTCAGTTC
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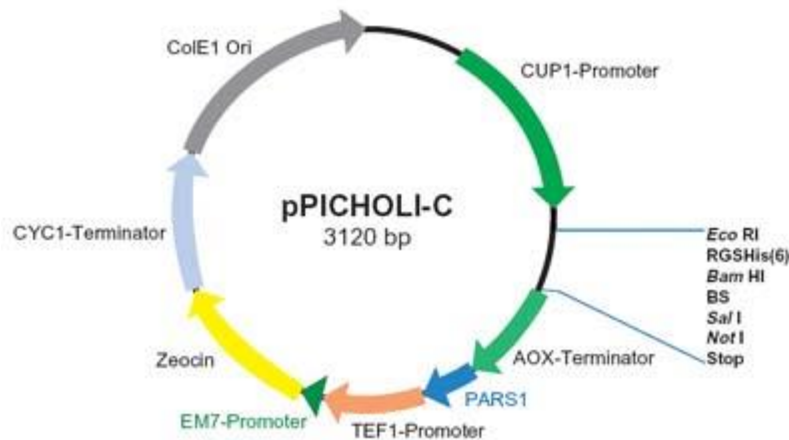
Sequence data of the cloning site of pPICHOLI-1: pPICHOLI-2 is lacking one of the G bases (dark shaded box) in front of the Sal restriction site, pPICHOLI-3 is lacking both G bases upstream SalI. The ATG start and the TAA stop codons are marked in bold and italic. *pPICHOLI-HA includes the 6xHis tag in front of the BamHI site and an HA tag ,instead of the biotinylation sequence, behind the BamHI site.

Note: BamHI is not a singular restriction site!



ColE1 ori: ColE1 origin; Zeocin: zeocin resistance; PARS1: *P. pastoris* autonomous replicating sequence; RGSHis(6): Poly(His)-Tag; BS: biotinylation sequence;

Note: BamHI is not a singular restriction site! pPICHOLI-C has no T7 promoter!

4. Advantages and Applications

1. Efficient, cost-effective, and time-saving protein production in either *E. coli* or *P. pastoris*
2. Problems like protein aggregation, denaturation, or accumulation in inclusion bodies may be avoided by choosing the *P. pastoris* expression system
3. Gene products toxic to *E. coli* may be easily expressed in *P. pastoris*
4. Vectors can be used for *in vitro* transcription/translation of cloned genes
5. Easy, fast, and reproducible purification of recombinant proteins via metal chelate affinity chromatography
6. Detection of recombinant proteins in immunoblotting experiments by commercially available antibodies
7. Biotinylation sequence provided by pPICHOLI-C allows rapid capture and screening assays
8. Production of functional target proteins useful as: (1) biotherapeutics or (2) for studying interactions with other biomolecules or drugs and structureactivity relationships or (3) for producing mono- and polyclonal antibodies as well as protein chips
9. Synthesis of proteins for crystallization and NMR analysis
10. Expression of proteins in high-throughput systems

5. Kit Components

PPICH	10 µg pPICHOLI-1 vector DNA
	10 µg pPICHOLI-2 vector DNA
	10 µg pPICHOLI-3 vector DNA
	10 µg pPICHOLI-C vector DNA
	10 µg pPICHOLI-HA vector DNA
	500 pmole AOX5'-Primer
	500 pmole AOX3'-Primer
	500 pmole CUP5'-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 (14, 15). Cloning of genes into the pPICHOLI dual expression vectors and expression/purification of recombinant proteins will be described here according to the inventors' protocols (16). In general, pPICHOLI offers two restriction sites for cloning: SalI and NotI, respectively. The cloning procedure/strategy requires an upstream primer containing a SalI site and a downstream primer containing a NotI site. These primers are as follows:

- the **5' primer (SalI):** 5'-AAAAG TCG ACC- first triplet following the ATG/translation initiation codon of your target gene - $(N)_{15–18}$-3' and

- the **3' primer (NotI)*:** 5'AAAA GCG GCC GC-TTA-$(N)_{15–18}$-3'.

The SalI and the NotI sites can be exchanged with XhoI, AvaI, and EagI sites. If the gene of interest contains internal SalI or NotI restriction sites, alternatively compatible cohesive ends can be generated using the enzymes XhoI or AvaI at the 5' end and EagI at the 3' end. The gene of interest is amplified using specific primers containing the required restriction sites, following restriction of the purified amplicon and ligation to the SalI/NotI or appropriately restricted vector. The transformation step requires an *E. coli* strain with high transformation efficiency, such as electrocompetent XL1Blue, DH5a, or SCS1. The designed primer must coincide with the open reading frame of the dual expression vector. Due to the reduced transformation efficiency (10^7-10^8 transformants/µg DNA) of the rubidium-competent BL21(DE3)pLysS, subcloning of the vector in an electrocompetent *E. coli* strain is recommended, following the manufacturer's instructions. When the transformants are confirmed (see 6.3.), the corresponding plasmid is isolated and transformed into the *E. coli* expression strain BL21(D3)pLysS and the *P. pastoris* expression strain, GS115 (his4, Mut^+; Life Technologies), for example.

*Please note that NotI requires long framing sequences for efficient cutting!

6.1. Strains

For subcloning strategies, common *E. coli* strains such as XL1Blue, DH5a, or SCS1 (Life Technologies; Stratagene) are used. Gene expression in *E. coli* using the dual expression vectors requires the *E. coli* strain BL21(DE3)pLysS (Novagen; Life Technologies) that carries the gene coding for phage T7 polymerase enabling T7 promoter induced transcription of the following cDNA.

Commonly used *P. pastoris* host strains are GS115 and KM71. The protease-deficient strain SMD1168 results in a marginal decrease in transformation efficiency and protein expression levels when compared to GS115.

6.2. Transformation protocols

6.2.1. *E. coli*

1. Electrocompetent cells, for example, XL1Blue (Stratagene), are prepared or obtained from the supplier, with a transformation efficiency of at least 10^9 transformants/µg DNA.
2. Strain BL21(DE3)pLysS (Novagen; Life Technologies).
3. 40% glucose in water; autoclaved or sterile filtered.
4. Antibiotic stock solution: 100 mg/mL zeocin; 34 mg/ml chloramphenicol.
5. Luria-Bertani (LB) medium per liter: 5 g yeast extract, 10 g NaCl, and 10 g bacto-tryptone. Adjust to pH 7.0 and autoclave. Add 15 g agar for plates. For selection and growth of transformants, add 250 µl of zeocin stock solution (25 µg/ml final concentration), and in the case of BL21(DE3)pLysS, add additional 1 ml chloramphenicol (34 µg/ml final concentration).
6. 100% glycerol, autoclaved.
7. TFBI: 30 mM KOAc, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% glycerol, pH 5.8, sterile filtered.
8. TFBII: 10 mM Na-MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol, pH 7.0, sterile filtered.

Preparation of *E. coli* BL21(DE3)pLysS competent cells and transformation

For transformation in the *E. coli* expression strain using a heat-shock method, competent cells of BL21(DE3)pLysS are prepared or obtained from the supplier (Novagen; Life Technologies), according to the following protocol:

1. Inoculate 50 ml 2YT medium (supplemented with 34 µg/ml chloramphenicol final concentration) with a fresh colony of BL21(DE3)pLysS from an agar plate, and grow overnight at 37 °C with shaking (250 rpm).
2. Inoculate 500 ml 2YT medium without antibiotics with the 5 ml overnight culture and grow it to an OD_{600} = 0.4 - 0.5 at 37 °C with shaking (250 rpm).
3. Cool the culture on ice for 20 minutes.
4. Harvest the culture by centrifugation at 1,300 g at 4 °C for 10 minutes, and resuspend the cells in 15 ml TFBI on ice.
5. Harvest the culture by centrifugation at 3,500 rpm at 4 °C for 10 minutes, and resuspend the cells in 4 ml TFBII on ice. The cells can be used directly for transformation or stored in 100 µl aliquots at -70 °C until use.

6. Dilute 0.1-0.5 µg DNA sample in 5-10 µl total volume of sterile distilled water, add 100 µl pre-cooled competent cells, and incubate on ice for 20 minutes.
7. Incubate cells in a 42 °C water-bath for 80 seconds.
8. Immediately add 1 ml fresh pre-warmed 2YT medium and regenerate the cells for 1 hour at 37 °C with shaking (250 rpm).
9. Spread aliquots onto agar plates containing 2YT medium, supplemented with 25 µg/ml zeocin and 34 µg/ml chloramphenicol, and incubate overnight at 37 °C (see Note 2).

6.2.2. *P. pastoris*

1. Zeocin stock solution: 100 mg/ml.
2. YPD medium: 10 g yeast extract and 10 g bacto-tryptone per liter, autoclave, and add 50 ml of sterile 40% glucose stock solution. For YPD plates: add 15 g agar per liter.
3. 5 M betaine in water, sterile filtered.
4. 100% glycerol, sterile filtered.
5. Sterile, distilled water, cooled to 4 °C.
6. 1 M HEPES, pH 8.0, sterile filtered.
7. 1 M DTT, sterile filtered.
8. 1 M sorbitol, autoclaved and cooled to 4 °C.
9. YPD agar plates supplemented with 100 µg/ml zeocin.

Preparation of *P. Pastoris* electrocompetent cells and transformation

For transformation in *P. pastoris* using electroporation, electrocompetent cells are prepared as described below. They can be used directly, or stored at -70 °C.

1. Inoculate 10 ml YPD medium with a single fresh colony of *P. pastoris* from an agar plate, and grow overnight at 30 °C with shaking (250 rpm).
2. Inoculate 500 ml YPD medium with the 10 ml overnight culture (OD_{600} = 0.1) and grow it to an OD_{600} = 1.3-1.5 at 30 °C with shaking (250 rpm).
3. Harvest the culture by centrifugation at 2,000 g at 4 °C for 10 minutes, and suspend the cells in 100 ml YPD supplemented with 20 ml HEPES and 2.5 ml 1 M DTT. Incubate the cells for 15 minutes at 30 °C without shaking.
4. Add cold water to 500 ml and harvest the cells by centrifugation at 2,000 g at 4 °C for 10 minutes.
5. Wash the cells with 250 ml cold water and collect the cells by centrifugation at 2,000 g at 4 °C for 10 minutes.
6. Wash the cells with 20 ml cold 1 M sorbitol and centrifuge at 2,000 g at 4 °C.
7. Resuspend the cells in 500 µl cold 1 M sorbitol. The cells can be used directly for transformation, or can be stored in aliquots at -70 °C until use.
8. Dilute 100 ng DNA sample in 5 µl total volume of sterile distilled water, add 40 µl competent cells and transfer into a 2 mm gap electroporation cuvette, precooled on ice.
9. Pulse cells according to the following parameters, when a Gene-Pulser (Bio-Rad) is being used: 1,500 V, 200 W, 25 µF. For other electroporation instruments, follow the manufacturer's recommendations with respect to yeast transformation.
10. Immediately add 1 ml cold 1 M sorbitol, transfer into a sterile 1.5 ml reaction tube and regenerate cells for at least 30 minutes at 30 °C with shaking.
11. Spread aliquots onto agar plates containing YPD, supplemented with 100 µg/ml zeocin, and incubate for two days at 30 °C (see Note 2). When using plasmids containing the PARS replicating sequence, a transformation efficiency of 10^5 transformants/µg DNA is expected.

6.3. Analysis of transformants

1. LB agar plate supplemented with 25 µg/ml zeocin.
2. YPD agar plate, supplemented with 100 µg/ml zeocin.
3. PCR mix: 50 mM KCl, 0.1% Tween 20, 1.5 mM $MgCl_2$, 35 mM Tris-Base, 15 mM Tris-HCl, pH 8.8, 0.2 mM dNTPs, 3 units Taq.
4. Primer:
   AOX5': TTGCGACTGG TTCCAATTGA CAAG; 10 pmol/µl.
   AOX3': CATCTCTCAG GCAAATGGCA TTCTG; 10 pmol/µl.
   CUP5': TGTACAATCA ATCAATCAAT CA; 10 pmol/µl.
5. Lyticase (e.g.: Sigma L2524) 6 mg/ml in water.

In general, transformants growing on selection medium of both *E. coli* and *P. pastoris* can be analyzed by PCR amplification of the specific gene insert using the same primer pair combination: AOX5' and AOX3' (pPICHOLI) or Cup5' and AOX3' (pPICHOLI-C), respectively. Due to the different stability and composition of the cell wall of *E. coli* and yeast, PCR amplification requires different conditions for cell disruption. *E. coli* cells are disrupted by heating (94 °C for 4 minutes) when the DNA is exposed for amplification, whereas the *P. pastoris* cell wall is enzymatically digested (zymolyase or lyticase at 37 °C for 30 minutes) leading to protoplasts which are more susceptible to heat or detergents. Then, following a heating step (94 °C for 4 minutes), DNA is exposed for amplification.

E. coli

1. Prepare a PCR mix (50 mM KCl, 0.1% Tween 20, 1.5 mM $MgCl_2$, 35 mM Tris-Base, 15 mM Tris-HCl, 0.2 mM dNTPs, 3 units Taq) sufficient for an appropriate number of transformants. To analyze, add 1 µl of each primer (AOX5' or CUP5'/AOX3'; 10 pmol/µl) for each transformant.
2. Distribute 30-50 µl per sample in PCR tubes.
3. With a toothpick, pick into a single colony and transfer the cells first onto a fresh LB agar plate supplemented with zeocin, and then into the corresponding PCR tube.
4. The agar plate is incubated at 37 °C overnight.
5. The PCR is performed under the following conditions: 4 minutes at 94 °C (1 cycle), 45 seconds at 94 °C, 20 seconds at 55 °C, and 80 seconds at 72 °C (24 cycles).
6. The PCR products are electrophoretically separated and analyzed.

P. pastoris

1. Prepare a PCR mix (50 mM KCl, 0.1% Tween 20, 1.5 mM $MgCl_2$, 35 mM Tris-Base, 15 mM Tris-HCl, 0.2 mM dNTPs, 3 units Taq) sufficient for an appropriate number of transformants to analyze and add 1 µl of each Primer (AOX5' or CUP5'/AOX3'; 10 pmol/µl) for each transformant.
2. Add 0.1 µg/µl lyticase per sample.
3. Dispense 30-50 µl/sample into PCR tubes.
4. With a toothpick, pick into a single colony and transfer the cells first onto a fresh YPD agar plate supplemented with zeocin, and then into the corresponding PCR tube.
5. The agar plate is incubated at 30 °C overnight.
6. The PCR is performed under the following conditions: 30 minutes at 37 °C, 4 minutes at 94 °C (1 cycle), 45 seconds at 94 °C, 20 seconds at 55 °C, and 2 minutes 30 seconds at 72 °C (30 cycles), 10 minutes at 72 °C (1 cycle).
7. The PCR products are electrophoretically separated and analyzed.

6.4. Protein expression and purification (see notes 3 & 5)

6.4.1. *E. coli* protein expression and lysis

1. LB medium, supplemented with 25 µg/ml zeocin and 34 µg/ml chloramphenicol.
2. 1 M IPTG.
3. Phosphate solution: 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0.
4. Lysis buffer: 50 mM Tris-HCl, 300 mM NaCl, pH 8.0, supplemented with 10 mM imidazole, 1 mM PMSF, 0.25 mg/ml lysozyme, 1 mg/ml RNase, and 1 mg/ml DNase.
5. QIAGEN buffer A: 6 M guanidine hydrochloride (Gn-HCl), 0.1 M NaH_2PO_4, 10 mM Tris-HCl, pH 8.0.

6.4.2. *P. pastoris* protein expression and lysis

1. YPD medium supplemented with 100 µg/ml zeocin.
2. YNB stock solution: dissolve 134 g yeast nitrogen base with ammonium sulfate and without amino acids (Difco) in 1 L water and autoclave.
3. 100% methanol (when using pPICHOLI).
4. 1 M $CuSO_4$, autoclaved (when using pPICHOLI-C).
5. Biotin stock solution: dissolve 20 mg biotin (Sigma B-4639) in 100 ml water and filter-sterilize.
6. 100 mM potassium phosphate buffer, pH 6.0: add 13.2 ml 1 M K_2HPO_4 and 86.8 ml 1 M KH_2PO_4 to 900 ml water and filter-sterilize.
7. BMMY medium: 10 g yeast extract, 10 g bacto-trypton in 700 ml water, autoclave, add 100 ml YNB stock solution, 2 ml biotin stock solution, 100 ml 100 mM potassium phosphate buffer, and 100 µg/ml zeocin.
8. Yeast nitrogen base with dextrose (YNBD) medium: Yeast Nitrogen Base (Difco: 0919-07-03) 6.7 g/L water, autoclave, and add 50 ml/L of filter-sterilized, or autoclaved, 40% (w/v) glucose.
9. Glass beads (size 0.5 mm; Sigma G-8772).
10. Lysis buffer: 50 mM Tris-HCl, 300 mM NaCl, pH 8.0, supplemented with 10 mM imidazole, 1 mM PMSF.

6.4.3. Native purification

1. Ni-NTA agarose.
2. 1 M imidazole.
3. Wash buffer: 50 mM Tris-HCl, 300 mM NaCl, pH 8.0 supplemented with 20 mM imidazole.
4. Elution buffer: 50 mM Tris-HCl, 300 mM NaCl, pH 8.0, supplemented with 250 mM imidazole.

6.4.4. Denatured purification

1. Ni-NTA agarose.
2. Buffer C: 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 6.3.
3. Buffer E: 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 4.5.

It is recommended that small-scale expression and purification is used to determine if the protein is expressed, and from which host the protein can be purified in a soluble state, in *E. coli* or *P. pastoris*, respectively. When the host and conditions are determined, large-scale expression and purification can be performed in order to produce sufficient amounts of proteins for subsequent applications. The quantities of solutions and material of the different scales are listed in Table 1.

Table 1

E. coli				***P. pastoris***		
Step	Small scale		Large scale	Small scale		Large scale
Inoculation	200 µl		20 ml	0.5-1 ml		50-200 ml
Induction	+ 1800 µl		+ 200 ml	+ 4 ml		+ 200-800 ml
	denat.	native		denat.	native	
Split	1 ml	1 ml		2.5 ml	2.5 ml	
Lysis Buffer	200 µl	200 µl	0.5-1 ml	200 µl	200 µl	1-5 ml
Ni-NTA	50 µl	50 µl	200 µl	20 µl	20 µl	50-100 µl
Wash Buffer	200 µl	200 µl	2 ml	200 µl	200 µl	2 ml
Elution Buffer	35 µl	35 µl	100 µl	35 µl	35 µl	100 µl

E. coli expression and lysis

1. Inoculate LB medium, supplemented with 2% glucose and 25 µg/ml zeocin, with a fresh colony of the transformant and grow overnight at 37 °C with shaking (200 rpm).
2. Inoculate fresh LB medium supplemented with 25 µg/ml zeocin with the overnight culture (10% final concentration of cell suspension) and grow at 37 °C with shaking to an OD_{600} = 0.6-1.0.
3. Add IPTG to a final concentration of 1 mM to induce protein expression, and grow at 37 °C with shaking for further 3-5 hours.
4. For evaluation of small-scale cultures, cultures are divided into two equal parts, harvested by centrifugation at 4,000 g at 4 °C and frozen for at least 20 minutes at -70 °C.
5. Thaw cell pellets. For evaluation, the two cell pellets from the small-scale culture are resuspended in either lysis buffer (native lysis) or QIAGEN buffer A (denatured lysis). Cell pellets of the large cultures are resuspended in the appropriate buffer, either lysis buffer or QIAGEN buffer A.
6. Cells resuspended in lysis buffer are lysed either at 4 °C overnight, or 30 minutes on ice, followed by sonication. Cells resuspended in QIAGEN buffer A are incubated at room temperature with shaking for at least 1 hour (see Note 3).
7. Lysates were cleared by centrifugation at 10,000 g for 10 minutes at 4 °C (native lysis) or at room temperature (denatured lysis).

***P. pastoris* expression and lysis (see Note 3)**

1. Inoculate YPD medium, supplemented with 100 µg/ml zeocin, with a fresh colony of the transformants and grow overnight with shaking (250 rpm) at 30 °C.
2. Inoculate fresh BMMY medium supplemented with 100 µg/ml zeocin (pPICHOLI) or YNBD (pPICHOLI-C), supplemented with 100 µg/ml zeocin and 40 mg/ml histidine, 1:10 with the overnight culture (10% final concentration of cell suspension), and grow at 30 °C with shaking to an OD_{600} = 1.0.
3. Add methanol to a final concentration of 0.5% (v/v) (pPICHOLI) or 0.1 mM $CuSO_4$ (pPICHOLI-C) to induce protein expression and grow at 30 °C with shaking (250 rpm) for 2-3 days (pPICHOLI) or 1-2 hours (pPICHOLI-C).
4. For evaluation of small-scale cultures, cultures are divided into two equal parts, harvested by centrifugation at 2000 g at 4 °C and then frozen for at least 20 minutes at -70 °C.
5. Thaw cell pellets. For evaluation, the two cell pellets of the small-scale culture are resuspended in either lysis buffer (native lysis) or QIAGEN buffer A (denatured lysis). Cell pellets of the large cultures are resuspended in the appropriate buffer, either lysis buffer or QIAGEN buffer A.
6. Add 0.5-1 volumes of glass beads and perform 5-7 cycles of 1 minute vortex, 1 minute incubation on ice.
7. The lysates are cleared by centrifugation at 10,000 g for 10 minutes at 4 °C (native lysis) or at room temperature (denatured lysis).

Native purification

1. Add Ni-NTA agarose to the lysate, mix gently and incubate on a rotary shaker for 1 hour at 4 °C. The appropriate volume of Ni-NTA depends partly on the expression level of the protein. Highly expressed proteins require more purification matrix, and for less expressed proteins the volume of Ni-NTA has to be reduced to ensure a good quality of purification.
2. Load the suspension of lysate and Ni-NTA slurry onto a column and collect flow-through.
3. Wash three times with wash buffer.
4. Elute the protein four times with buffer E and collect flow-through fractions. Fractions can be analyzed by SDS-PAGE and Western blot. To increase protein concentration, and to decrease the elution volume, only one volume may be applied and incubated for 10 minutes onto the column without flow-through.

Denatured purification

1. Add Ni-NTA agarose to the lysate, mix gently, and incubate on a rotary shaker for 1 hour at room temperature. The appropriate volume of Ni-NTA depends partly of the expression level on the protein. Highly expressed proteins require more purification matrix, and for less expressed proteins the volume of Ni-NTA has to be reduced to ensure a good quality of purification.
2. Load the suspension of lysate and Ni-NTA slurry onto a column and collect flow-through.
3. Wash three times with buffer C.
4. Elute the protein four times with buffer E and collect flow-through fractions. Fractions can be analyzed by SDS-PAGE and Western blot. To increase protein concentration,

and to decrease the elution volume, only one volume may be applied and incubated for 10 minutes onto the column without flow-through.

Notes

1. When expressing proteins in *E. coli* using this system, it is important to use an *E. coli* strain that contains a T7 promoter, such as BL21(DE3)pLysS.
2. It is also important to note the difference in the concentration of zeocin antibiotic used in the different expression systems. In *E. coli*, less is used (25 µg/ml) as in *Pichia* (100 µg/ml zeocin). In addition, when the *E. coli* strain BL21 is used for expression, it is necessary to add 34 µg/ml chloramphenicol for selection of pLys.
3. For protein purification in *E. coli*, the lysate should be ultrasonicated longer. Otherwise, the lysate is mucilaginous and may clog the purification column.
4. For planning yeast protein expression experiments, it is important to note that AOX promoter is used. This is because the yeast transformants require 2 days to grow (*E. coli* transformants: 1 day) and the induction takes at least 2 days (again with *E. coli* or using pPICHOLI-C with the CUP1 promoter, it is only 1 day).

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8. Order Information, Shipping, and Storage

Order#	Product	Quantity
PPICH	pPICHOLI vectors DNA	5 x 10 µg

Shipped at room temperature (RT). Lyophilized plasmid DNA can be stored at 4 °C. Once the DNA has been dissolved in sterile water or buffer we recommend storage at -20 °C.

9. Contact and Support

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