pPICHOLI Shuttle Vector System

Order # PPICH

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Contents

1. Features

- Efficient, cost-effective, and time-saving protein production in either *E. coli* or *P. pastoris*
- Gene products toxic to *E. coli* may be easily expressed in *P. pastoris*
- No further cloning step needed: switch from *E.coli* expression to *P.pastoris* expression without changing the vector. Expression with *P. pastoris* may help to increase the protein yield by avoiding aggregation, denaturation, or formation of inclusion bodies of your protein of interest.
- Vectors can be used for *in vitro* transcription/translation of cloned genes
- Easy, fast, and reproducible purification of recombinant proteins via metal chelate affinity chromatography (His-tag)
- Detection of recombinant proteins in immunoblotting experiments by commercially available antibodies
- Biotinylation sequence provided by pPICHOLI-C allows rapid capture and screening assays
- 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
- Synthesis of proteins for crystallization and NMR analysis
- Expression of proteins in high-throughput systems

2. 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: 5’ TTGCGACTGG TTCCAATTGA CAAG 3’
	500 pmole AOX3'-Primer: 5’ CATCTCTCAG GCAAATGGCA TTCTG 3’
	500 pmole CUP5'-Primer: 5’ TGTACAATCA ATCAATCAAT CA 3’

Shipped at room temperature (RT), store at 4 °C. Once the DNA has been dissolved in sterile water or buffer we recommend storage at -20 °C.

3. 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, heterologous eukaryotic proteins expressed are often not

correctly folded or modified, and might form 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 have been constructed for this purpose. They enable 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).

4. The pPICHOLI Shuttle Vector System

The pPICHOLI Shuttle vector system is a dual expression system with many advantages: Expression with *E. coli* is simple to handle and allows a cost-effective and high-level production of heterologous proteins. Further, the pPICHOLI vector system is also a powerful eukaryotic tool and can be used for expression with *P. pastoris.* Here it shows rapid cell growth at high densities combined with high expression rates using the strong AOX or CUP1 promoter, respectively. It is ideally suited for expression of soluble proteins with post-translational modifications and proteins causing problems when expressed in *E. coli* (e.g., proteins toxic to *E. coli*). Transformation efficiency is high (6) and recovery of plasmids from *P. pastoris* is easy to handle

The pPICHOLI Expression Vectors
The pPICHOLI vectors have been designed for heterologous gene expression in the prokaryote *Escherichia coli* as well as in the yeast *Pichia pastoris*. The vectors contain:

- an inducible *E. coli* T7 promoter (exception: pPICHOLI-C has no T7 promoter!)
- an inducible yeast alcohol oxidase (AOX) promoter (exception: *pPICHOLI-C = CUP1 promoter*)

- sequences for autonomous replication in *E. coli* (ColE1 incompatibility group) and *P. pastoris* (PARS1)
- selection marker: Zeocin
- a double tag consisting of Arg-Gly-Ser(His)$_6$ tag and an *in vivo* biotinylation sequence (exception: pPICHOLI-HA lacks the biotin-tag, but instead possesses an HA (hemagglutinin) epitope
- a multiple cloning site (MCS) that enables convenient ligation of DNA fragments into the vectors

pPICHOLI-1, pPICHOLI-2, pPICHOLI-3

For expression in yeast, the pPICHOLI vectors contain the strong alcohol oxidase promoter (AOX; functional in yeast) that is tightly regulated. Protein expression is completely repressed when grown on glucose and maximally induced when grown on methanol (10). 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 expression vectors include a double tag consisting of Arg-Gly-Ser(His)$_6$ tag 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 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 bp) and pPICHOLI-3 (3577 bp), respectively, are lacking one or both of these G bases (s. p.9, Fig1a). For replication in Pichia, all vectors own the *Pichia*-specific autonomous replicating sequence (PARS1). Replication in E.coli is ensured by an origin of replication from the ColE1 incompatibility group. Due to the use of a common selection marker zeocin, the sizes of the shuttle vectors remain small, hence they remain convenient for handling, cloning, and transformation.

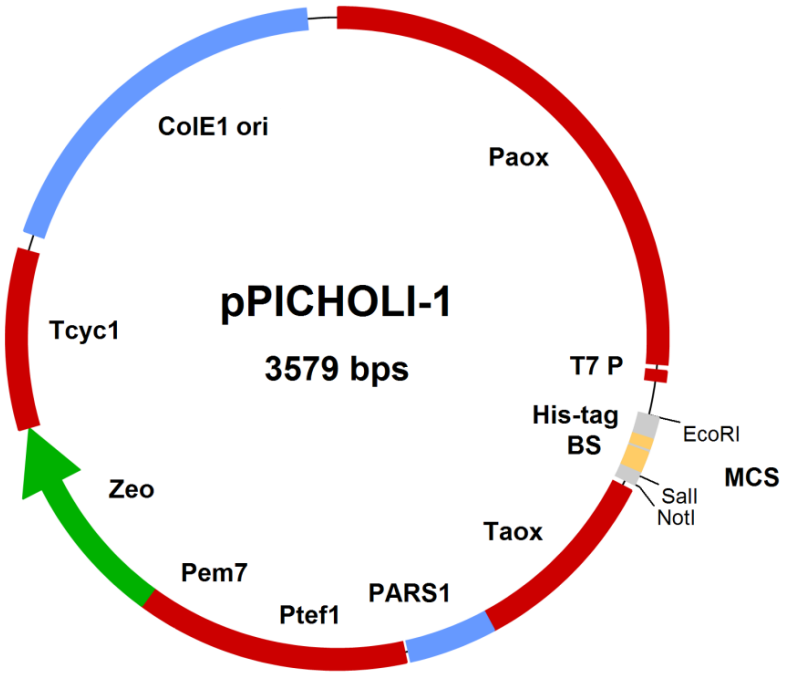
pPICHOLI-HA

pPICHOLI-HA (3568 bp) lacks the biotin-tag, but instead possesses an HA (hemagglutinin) epitope. Apart from that it contains all other features of the pPICHOLI-1, -2, 3 vectors.

pPICHOLI-C

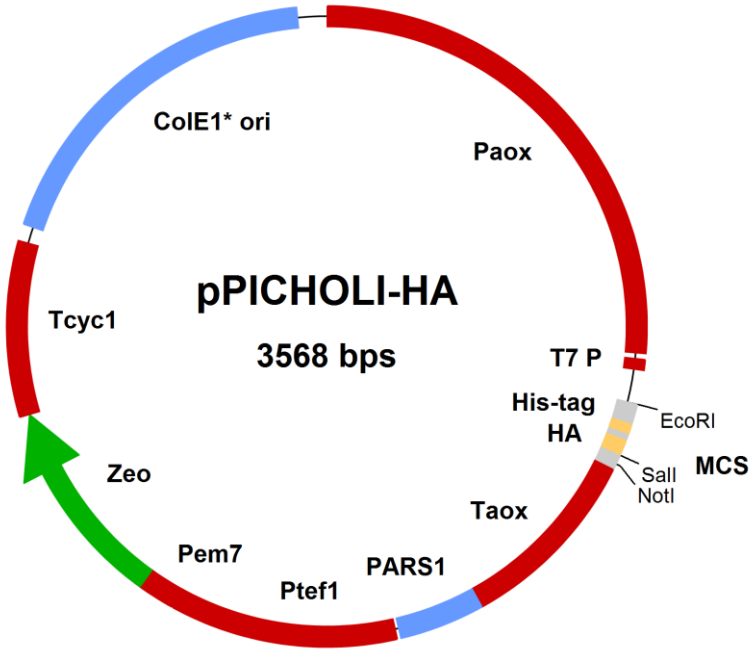
Like pPICHOLI-3; Instead of the AOX promoter pPICHOLI-C (3120 bp) carries the copper-inducible CUP1 promoter of *Saccharomyces cerevisiae* which has been shown to reduce the induction time greatly (11, 12).It has no T7 promoter and is not suited for expression in *E. coli*.

4.1. Vector map pPICHOLI-1



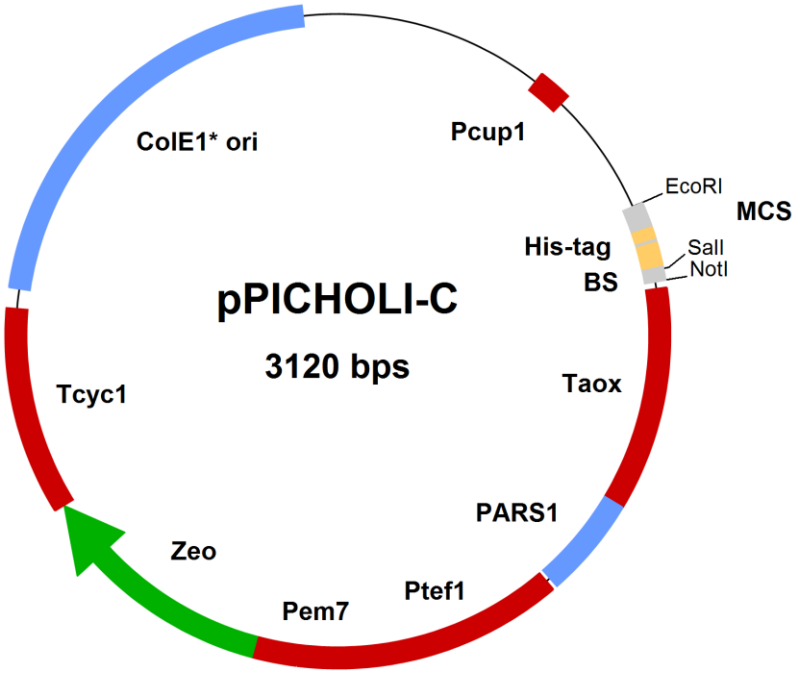
	Type	Start	End	Name	Description
	Promoter	1	940	Paox	AOX promoter
	Promoter	951	970	T7 P	T7 promoter
	Region	1032	1155	MCS	Multiple Cloning Site
	Tag	1062	1088	His tag	RGS (His)6 tag
	Tag	1095	1130	BS	Biotinylation sequence
	Terminator	1166	1507	Taox	AOX terminator
	Origin of replication	1508	1662	PARS	Autonomous replicating sequence (Pichia)
	Promoter	1669	2079	Ptef1	TEF1 promoter
	Promoter	2081	2158	Pem7	EM7 promoter
	Selectable genetic marker	2149	2523	Zeo	Zeocin resistance gene
	Terminator	2524	2841	Tcyc1	CYC1 terminator
	Origin of replication	2872	3525	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group

Vector map pPICHOLI-HA



	Type	Start	End	Name	Description
	Promoter	1	940	Paox	AOX promoter
	Promoter	951	970	T7 P	T7 promoter
	Region	1032	1155	MCS	Multiple Cloning Site
	Tag	1062	1088	His tag	RGS (His)6 tag
	Tag	1098	1126	HA	Hemagglutinin epitope
	Terminator	1155	1496	Taox	AOX terminator
	Origin of replication	1497	1651	PARS1	Autonomous replicating sequence (Pichia)
	Promoter	1658	2068	Ptef1	TEF1 promoter
	Promoter	2070	2137	Pem7	EM7 promoter
	Selectable genetic marker	2138	2512	Zeo	Zeocin resistance gene
	Terminator	2513	2830	Tcyc1	CYC1 terminator
	Origin of replication	2861	3514	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group

4.2. Vector map pPICHOLI-C



	Type	Start	End	Name	Description
	Promoter	328	380	Pcup1	CUP1 promoter
	Region	575	696	MCS	Multiple Cloning Site
	Tag	605	631	His tag	RGS (His)6 tag
	Tag	1098	1126	BS	Biotinylation sequence
	Terminator	707	1048	Taox	AOX terminator
	Origin of replication	1049	1203	PARS1	Autonomous replicating sequence (Pichia)
	Promoter	1210	1620	Ptef1	TEF1 promoter
	Promoter	1622	1689	Pem7	EM7 promoter
	Selectable genetic marker	1690	2064	Zeo	Zeocin resistance gene
	Terminator	2065	2382	Tcyc1	CYC1 terminator
	Origin of replication	2413	3066	ColE1 ori*	Origin of replication (*E. coli*); ColE1 incompatibility group

4.3. Multiple Cloning Sites of pPICHOLI Vectors

a)

```
                                                                    R   G   S
CAATTTCACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCGCATCAC
          EcoRI
     6x His tag                                  Biotinylation Sequence
CATCACCATCACGGATCCCTGAACGACATCTTCGAAGCTCAGAAAATCGAATGGAA

AAAGGGTCGACCTGCAAGATCTGCGGCCGCGAATTAATTCGCCTTAGA
      SalI                    NotI
```

b)

```
                                                                R   G   S
CAATTTCACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCAT
          EcoRI
     6x His tag                          Hemagglutinine Epitope
CACCATCACCATGGGATCCGCTACCCATACGACGTCCCAGACTACGCTGGGTCGAC
                                                          SalI

CTGCAAGATCTGCGGCCGCGAATTAATTCGCCTTAGA
          NotI
```

c)

```
                                                                R   G   S
CAATTTCACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCGCATCAC
          EcoRI
     6x His tag                          Biotinylation Sequence
CATCACCATCACGGATCCCTGAACGACATCTTCGAAGCTCAGAAAATCGAATGGAA

AAAGTCGACCTGCAAGATCTGCGGCCGCGAATTAATTCGCCTTAGA
      SalI                    NotI
```

Fig.1: Multiple Cloning Sites of pPICHOLI vectors

The ATG start and the TAA stop codons are marked in bold, tag sequences are marked in yellow.

a) pPICHOLI-1: The sequence of the Multiple Cloning Site of **pPICHOLI-1** including the RGS(His)$_6$ tag and the biotinylation signal is shown. **pPICHOLI-2** is lacking one of the G bases (green box) upstream of the SalI restriction site, **pPICHOLI-3** is lacking both G bases upstream of SalI site.

b) pPICHOLI-HA: The sequence of the Multiple Cloning Site of **pPICHOLI-HA** including the RGS(His)$_6$ tag and the HA epitope is shown.

c) pPICHOLI-C: The sequence of the Multiple Cloning Site of **pPICHOLI-C** including the RGS(His)$_6$ tag and the biotinylation signal is shown.

5. Protocols

5.1. Storage and Handling Instructions

Plasmids and primers are supplied lyophilized. To each plasmid, add 100 µl distilled water (final concentration 0.1 µg/µl) and incubate at 50 °C for 5 minutes. Vortex for 1 minute and store at -20 °C. Primers should be dissolved in 50 µl distilled water (final concentration 10 pmol/µl) and stored at -20 °C.

5.2. Cloning Strategy

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 should be 15-18 bp in size and must be adapted to the target gene of interest. Therefore these primers are not included in this kit.

Recommended cloning primers:

5' primer (SalI):

5'-AAAAG TCG ACC- XXX - $(N)_{15–18}$-3'

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

XXX: first triplet following the ATG/translation initiation codon of your target gene and
N: nucleotides fitting to your gene of interest

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, 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^+), for example.

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

5.3. Strains

For subcloning strategies, common *E. coli* strains such as XL1Blue, DH5a, or SCS1 are recommended. Gene expression in *E. coli* using the dual expression vectors requires the *E. coli* strain BL21(DE3)pLysS that carries the gene encoding for phage T7 polymerase enabling T7 promoter induced transcription.

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.

5.4. Growth Conditions

Detailed protocols for *E. coli* molecular genetic handling (growth, transformation, etc.) can be found in the relevant laboratory manuals such as Sambrook and Russell (2001).
E. coli can be grown aerobically at 37 °C in 2xYT medium (Bagyan *et al*., 1998). Under optimal conditions the doubling time of *E. coli* is 20 min. *P. pichia* cells are grown in YPD medium, with shaking (250 rpm) at 30 °C. For selecting transformed *E. coli* cells use 25 µg/ml zeocin., for *P. pichia* use 100 µg/ml zeocin. In addition, when the *E. coli* strain BL21(DE3)pLysS is used for expression, it is necessary to add 34 µg/ml chloramphenicol for selection of pLysS.

5.5. Transformation of *P. pichia*

For transforming *P. pastoris* by 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 1M HEPES (pH8) and 2.5 ml 1 M DTT. Incubate the cells for 15 minutes at 30 °C without shaking.
4. Add distilled 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 distilled 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 notes below). When using plasmids containing the PARS replicating sequence, a transformation efficiency of 10^5 transformants/µg DNA is expected.

5.6. Analysis of Transformants

E. coli and *P. pastoris* transformants growing on selection medium can be analyzed by Colony PCR amplification of the specific gene insert using the 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 only by heating (94 °C for 4 minutes), whereas *P. pastoris* cell walls are primarily enzymatically digested (zymolyase or lyticase (6mg/ml) at 37 °C for 30 minutes) followed by heat treatment of the cells (94 °C for 4 minutes).

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.

5.7. Protein expression and purification

It is recommended that small-scale expression and purification is used first 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* protein 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 A (native lysis) or Lysis buffer B (denatured lysis). Cell pellets of the large cultures are resuspended in the appropriate buffer, either Lysis buffer A or Lysis buffer B.

6. Cells resuspended in Lysis buffer A are lysed either at 4 °C overnight, or 30 minutes on ice, followed by sonication. Cells resuspended in Lysis buffer B 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* protein expression and lysis**

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 A (native lysis) or Lysis buffer B (denatured lysis). Cell pellets of the large cultures are resuspended in the appropriate buffer, either Lysis buffer A or Lysis buffer B.
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 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 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).

5.8. Media and Solutions

LB Medium:	10 g tryptone 5 g yeast extract 10 g sodium chloride (NaCl) add distilled water to 1000 ml and autoclave
LB agar plates	add 1.5 % agar to the LB medium before autoclaving
YPD Medium:	10 g yeast extract 10 g tryptone per liter, add distilled water to 1000 ml and autoclave, then add 50 ml of sterile 40% glucose stock solution.

YPD agar plates:	add 1,5 % agar to the YPD medium before autoclaving.
YNB Stock Solution:	134 g yeast nitrogen base with ammonium sulfate and without amino acids add distilled water to 1000 ml and autoclave
BMMY Medium:	10 g yeast extract, 10 g bacto-trypton Add distilled water to 700 ml, autoclave, Then add 100 ml YNB stock solution, 2 ml biotin stock solution, 100 ml 100 mM potassium phosphate buffer, 100 µg/ml zeocin
Biotin Stock Solution:	dissolve 20 mg biotin in 100 ml water, filter-sterilize
Potassium Phosphate buffer 100 mM, pH 6.0	13.2 ml 1 M K_2HPO_4 86.8 ml 1 M KH_2PO_4 add to 900 ml water, filter-sterilize.
YNBD Medium:	Yeast Nitrogen Base 6.7 g/L water, autoclave, and add 50 ml/L of filter-sterilized, or autoclaved, 40% (w/v) glucose.
Phosphate Solution:	50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0.
Lysis buffer A:	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.
Lysis buffer B:	6 M guanidine hydrochloride (Gn-HCl), 0.1 M NaH_2PO_4, 10 mM Tris-HCl, pH 8.0.

Wash buffer:	50 mM Tris-HCl, 300 mM NaCl, pH 8.0 supplemented with 20 mM imidazole.
Elution buffer:	50 mM Tris-HCl, 300 mM NaCl, pH 8.0, supplemented with 250 mM imidazole.
Buffer C:	8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 6.3
Buffer E:	8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 4.5.

6. References

1. Baneyx, F. (1999) Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol*. **10**, 411–421.
2. Hannig, G. and Makrides, S. C. (1998) Strategies for optimizing heterologous protein expression in *Escherichia coli*. *Trends Biotechnol*. **16**, 54– 560.
3. Makrides, S. C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol. Rev*. **60**, 512–38.
4. Cregg, J. M., Vedvick, T. S., and Raschke, W. C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology* **11**, 905–910.
5. Faber, K. N., Harder, W., Ab, G., and Veenhuis, M. (1995) Review: methylotropic yeasts as factories for the production of foreign proteins. *Yeast* **11**, 1331–1344.
6. Faber, K. N., Westra, S., Waterham, H. R., Keizer, G. I., Harder, W., and Veenhuis, G. A. (1996) Foreign gene expression in Hansenula polymorpha. A system for the synthesis of small functional peptides. Appl. *Microbiol. Biotechnol*. **45**, 72–79.
7. Romanos, M. A., Scorer, C. A., and Clare, J. J. (1992) Foreign gene expression in yeast: a review. *Yeast* **8**, 423–488.
8. Lueking, A., Holz, C., Gotthold, C., Lehrach, H., and Cahill, D. (2000) A system for dual protein expression in *Pichia pastoris* and *Escherichia coli*. *Protein Expr. Purif*. **20**, 372–378.
9. Cereghino, J. L. and Cregg, J. M. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol. Rev*. **24**, 45–66.
10. Tschopp, J. F., Brust, P. F., Cregg, J. M., Stillman, C. A., and Gingeras, T. R. (1987) Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Res*. **15**, 3859–3876.
11. Macreadie, I. G., Horaitis, O., Verkuylen, A. J., and Savin, K. W. (1991) Improved shuttle vectors for cloning and high-level Cu(2+)-mediated expression of foreign genes in yeast. *Gene* **104**, 107–111.
12. Koller, A., Valesco, J., and Subramani, S. (2000) The CUP1 promoter of *Saccharomyces cerevisiae* is inducible by copper in *Pichia pastoris*. *Yeast* **16**, 651–656.

13. Schatz, P. (1993) Use of peptide libraries to map the substrate specifity of a peptide-modifying enzyme: A 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. *Biotechnology* **11**, 1138–1143.
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: a laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbory, NY.
15. Ausubel, F. M., Brent, R., Kingston, R.E., Moore, D. D., Seidman, J.G., Smith, J. A. and Struhl, K. (2000) *Current Protocols in Molecular Biology*, vols 1 and 2, J. Wiley & Sons, New York.
16. Lueking, A., Horn, S., Lehrach, H., and Cahill, D.J. (2003) A dual-expression vector allowing expression in *E. coli* and *P. pastoris*. *Methods Mol. Biol*. **205**, 31–42.

7. 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.

8. Contact and Support

MoBiTec GmbH ● Lotzestrasse 22a ● D-37083 Goettingen ● Germany

Customer Service – General inquiries & orders
phone: +49 (0)551 707 22 0
fax: +49 (0)551 707 22 22
e-mail: order@mobitec.com

Technical Service – Product information
phone: +49 (0)551 707 22 70
fax: +49 (0)551 707 22 77
e-mail: info@mobitec.com

MoBiTec in your area: Find your local distributor at www.mobitec.com