CHAPTER TWELVE

Protein Expression-Yeast

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Contents

Abstract

Yeast is an excellent system for the expression of recombinant eukaryotic proteins. Both endogenous and heterologous proteins can be overexpressed in yeast (Phan et al., 2001; Ton and Rao, 2004). Because yeast is easy to manipulate genetically, a strain can be optimized for the expression of a specific protein. Many eukaryotic proteins contain posttranslational modifications that can be performed in yeast but not in bacterial expression systems. In comparison with mammalian cell culture expression systems, growing yeast is both faster and less expensive, and large-scale cultures can be performed using fermentation. While several different yeast expression systems exist, this

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chapter focuses on the budding yeast *Saccharomyces cerevisiae* and will briefly describe some options to consider when selecting vectors and tags to be used for protein expression. Throughout this chapter, the expression and purification of yeast eIF3 is shown as an example alongside a general scheme outline.

1. THEORY

Depending on the specific protein(s) to be expressed, or more often overexpressed, several different factors must be considered:

- If the protein is toxic to cell growth when overexpressed or has to be modified in the cell by a limiting factor, a single-, or low-copy plasmid should be used for expression.
- In many cases the amount of the expressed protein is the most important factor and therefore high-copy (2 μm) vectors are routinely used for overexpression.
- If the protein of interest is an endogenous protein, its own promoter can be used for the expression. This is achieved by cloning a piece of DNA containing the promoter region and the sequence encoding the protein together with the terminator into a 2 μm vector.

  In the example presented here, eIF3 is composed of five essential subunits that are overexpressed from their native promoters using two 2 μm vectors. One plasmid contains the LEU2 selectable marker and three of the five genes and the second plasmid contains the URA3 selectable marker and the remaining two genes. The yeast strain used for expression of the five subunits has two proteases deleted (Pep4 and Prb1) to reduce degradation during purification (Phan et al., 2001).
- If proteins from another species are to be expressed, the DNA encoding the protein must be cloned downstream of an endogenous promoter. A housekeeping promoter will normally give a constitutive expression of the protein. However, if the protein of interest is toxic to cell growth or only low amounts can be obtained from a housekeeping promoter, an inducible promoter should be used instead. Examples of both are as follows:

  Constitutive promoters:

  PGK1 (phosphoglycerate kinase-1)
  ADH1 (alcohol dehydrogenase-1)
  GPD (glyceraldehyde-3-phosphate dehydrogenase)
  CYC1 (Cytochrome c)

Regulatable promoters:

GAL1-10 (induced by galactose)

MET25 (methionine-responsive)

CUP1 (copper-dependent)

The last two promoters have variable expression in response to the regulator.

For further information regarding vector systems, see Funk et al. (2002), which thoroughly describes a variety of different promoters and selective markers on either single- or high-copy vectors.

Purification can often be facilitated by tagging the protein of interest. Tag purifications can, in some cases, result in pure protein after only one purification step. However, a tag can also be used to make an otherwise insoluble protein soluble. Depending on potential interactions with other proteins, a tag may be added to the N-terminus or C-terminus to avoid steric hindrance.

Commonly used tags for affinity purification:

His (6-10 histidines)

GST (glutathione-S-transferase)

FLAG (contains 8 amino acids; DYKDDDDK)

HA (hemagglutinin epitope)

MBP (maltose-binding protein)

CBP (calmodulin-binding protein)

Commonly used tags for increasing solubility:

MBP (maltose-binding protein)

SUMO (small ubiquitin modifier)

GST (glutathione-S-transferase)

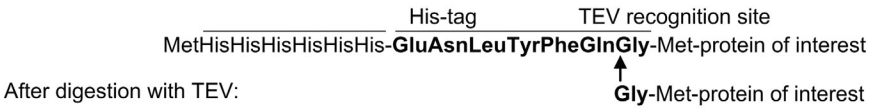
In the example presented here for purification of eIF3, one subunit (Prt1) has a His_8 tag at its N-terminus allowing Ni^{2+} affinity purification to be performed. Purification of eIF3 using different epitope tags is described in Nielsen and Valásek (2007).

For further information regarding tagging proteins, see Malhotra (2009), which thoroughly describes several tags commonly used for both solubility and affinity purification.

In some cases, particularly when large tags such as GST or MBP have been used for purification, it may be necessary to remove the tag before experiments using the purified protein can be conducted. The insertion of a cleavage site for a specific endoprotease between the tag and the start of the protein will enable the proteolytic removal of the tag.

Commonly used proteases:
TEV (Tobacco Etch Virus)
Enterokinase
Factor Xa
Thrombin

For further information regarding proteases, see Malhotra (2009). The TEV protease can be made in-house (Tropea et al., 2009). If the cleavage site is inserted at the N-terminal end of the protein of interest, a single glycine immediately upstream of the start methionine will remain after proteolytic cleavage (see below). Cleavage occurs between the glutamine and glycine (arrow below the TEV recognition site). Purifying the TEV protease in-house, using a noncleavable His-tag, enables easy removal of the TEV protease. This is done by loading the cleaved dialyzed protein of interest, together with the His-tagged TEV protease, on a HisTrap nickel column and collecting the flow through. The flow through should only contain the cleaved protein of interest, while the His-tagged TEV protease and the cleaved His-tag should remain bound to the nickel column.



Two different approaches to breaking open yeast cells are described. The first method uses a high-pressure homogenizer (HPH EmulsiFlex-C5 manufactured by AVESTIN). This method requires the cell mass to be resuspended in liquid. After breaking open the yeast cells, the extract should be used immediately for further purification. The second approach uses a Freezer/Mill 6870 from SPEX. Freezer/Mills are cryogenic laboratory mills that cool samples to cryogenic temperatures and pulverize them by magnetically shuttling a steel impactor back and forth against two stationary end plugs. In addition to keeping the temperature at a minimum, the Mill allows the cells to be added as droplets (see below) and the powder generated by the Mill can be stored directly at −80 °C. Use of the Freezer/Mill is preferred if available.

2. EQUIPMENT

−80 °C freezer
Freezer/Mill or a High-pressure homogenizer (HPH)

Refrigerated centrifuge (Sorvall RC-3C Plus or similar)
H-6000B rotor (Sorvall) or similar (6 × 1 l capacity)
Heater incubator-shaker
Polyacrylamide gel electrophoresis equipment
FPLC or peristaltic pump
UV/vis spectrophotometer
Micropipettors
Micropipettor tips
25-ml disposable serological pipettes
Petri plates
1.5-ml microcentrifuge tubes
1-l centrifuge bottles
5-l baffled flasks
Cheesecloth (if using HPH)
Filter units (5 and 0.8 μm)
HisTrap nickel column (GE Healthcare)
Amicon Ultra concentrator (10 K MWCO) (Millipore)
Superose 12 gel filtration column
Phosphocellulose column
Dialysis tubing

3. MATERIALS

Bacto-yeast extract
Bacto-Peptone
Bacto-agar
Bacto-yeast nitrogen base w/o amino acids
Potassium chloride (KCl)
Potassium acetate (KOAc)
Glycerol
Glucose
Drop-out amino acid mix
Magnesium chloride ($MgCl_2$)
Dithiothreitol (DTT)
Imidazole
HEPES
Potassium hydroxide (KOH)
Sodium dodecyl sulfate (SDS)
EDTA

β-Mercaptoethanol
Protease inhibitors
Coomassie blue

3.1. Solutions & buffers

Step 1 YPD

Component	Final concentration	Amount
Bacto-yeast extract	1%	10 g
Bacto-Peptone	2%	20 g
Glucose	2%	20 g

Add water to 1 l

Synthetic Complete (SC) drop-out media

Component	Final concentration	Amount
Bacto-yeast nitrogen base w/o amino acids	0.67%	6.7 g
Glucose	2%	20 g
Drop-out mix	0.2%	2 g

Add water to 1 l
For plates, add 20 g of Bacto-agar per liter. If volumes are larger than 1 l, autoclave salts, glucose, and agar separately

NCLB buffer

Component	Final concentration	Stock	Amount
Hepes-KOH, pH 7.6	20 mM	1 M	20 ml
KCl	350 mM	2 M	175 ml
$MgCl_2$	5 mM	1 M	5 ml
Imidazole	20 mM	4 M	5 ml
Glycerol	10%	100%	100 ml
β-Mercaptoethanol	10 mM	14 M	0.71 ml
Protease inhibitors			

Add water to 1 l

Step 3 NCEB buffer

Component	Final concentration	Stock	Amount
Hepes–KOH, pH 7.6	20 mM	1 M	2 ml
KCl	350 mM	2 M	17.5 ml
$MgCl_2$	5 mM	1 M	0.5 ml
Imidazole	250 mM	4 M	6.25 ml
Glycerol	10%	100%	10 ml
β-Mercaptoethanol	10 mM	14 M	71 μl
Protease inhibitors			

Add water to 100 ml

Low salt buffer

Component	Final concentration	Stock	Amount
Hepes–KOH, pH 7.6	20 mM	1 M	20 ml
KCl	100 mM	2 M	50 ml
EDTA	0.1 mM	500 mM	0.2 ml
Glycerol	10%	100%	100 ml
DTT	2 mM	1 M	2 ml

Add water to 1 l

200 mM KCl buffer

Component	Final concentration	Stock	Amount
Hepes–KOH, pH 7.6	20 mM	1 M	2 ml
KCl	200 mM	2 M	10 ml
EDTA	0.1 mM	500 mM	20 μl
Glycerol	10%	100%	10 ml
DTT	2 mM	1 M	0.2 ml

Add water to 100 ml

350 mM KCl buffer

Component	Final concentration	Stock	Amount
Hepes–KOH, pH 7.6	20 mM	1 M	2 ml
KCl	350 mM	2 M	17.5 ml
EDTA	0.1 mM	500 mM	20 µl
Glycerol	10%	100%	10 ml
DTT	2 mM	1 M	0.2 ml

Add water to 100 ml

1 M KCl buffer

Component	Final concentration	Stock	Amount
Hepes–KOH, pH 7.6	20 mM	1 M	2 ml
KCl	1 M	2 M	50 ml
EDTA	0.1 mM	500 mM	20 µl
Glycerol	10%	100%	10 ml
DTT	2 mM	1 M	0.2 ml

Add water to 100 ml

Enzyme storage buffer

Component	Final concentration	Stock	Amount
Hepes–KOH, pH 7.6	20 mM	1 M	2 ml
KOAc, pH 7.6	100 mM	1 M	10 ml
Glycerol	10%	100%	10 ml
DTT	2 mM	1 M	0.2 ml

Add water to 100 ml

Tip	*Print out recipes of all stock solutions needed to create the buffers.*

4. PROTOCOL

4.1. Duration

Preparation	About 1 day
Protocol	About 7–8 days

4.2. Preparation

Prepare plates and media.

If the plasmid(s) have to be transformed into the yeast strain please refer to one of the following references (Gietz and Schiestl, 2007a; b).

4.3. Tip

The protocol can be paused at various times as indicated. The material should be stored at −80 °C.

See Fig. 12.1 for the flowchart of the complete protocol.

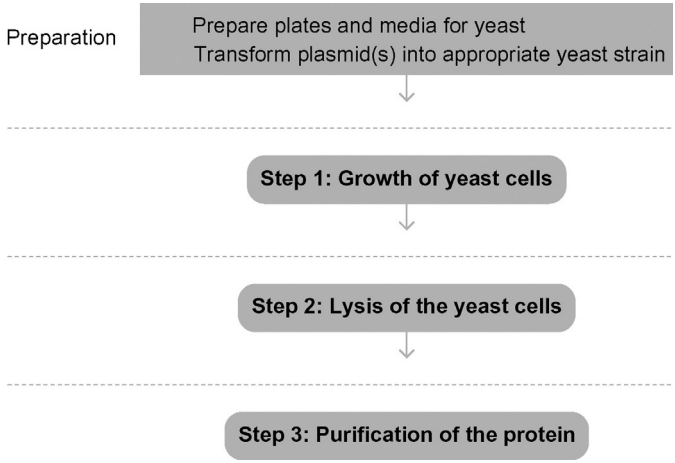


Figure 12.1 Flowchart of the complete protocol, including preparation.

5. STEP 1 GROWTH OF YEAST CELLS

5.1. Overview

A starter culture, made from freshly streaked plates, is used to inoculate the larger cultures. Cells are collected and resuspended in an appropriate buffer.

5.2. Duration

4–5 days

1.1 From a glycerol stock, streak the yeast cells on appropriate plates and incubate at 30 °C for 2–3 days.

1.2 Inoculate an overnight starter culture in ~12 ml selective medium, preferably selective SC medium. Incubate at 30 °C with shaking (250 rpm).

1.3 Inoculate six 5-l baffled flasks containing 2 l SC selective medium or YPD medium with 2 ml starter culture per flask. Grow overnight (~16 h) at 30 °C shaking at 250 rpm.

1.4 Spin down the cells using a centrifuge with 1 l capacity bottles (Sorvall RC3C plus or similar) at 4200 rpm, 4 °C for 30 min and resuspend the cells in buffer according to the method used for breaking open the yeast cells.

- For the HPH, use 2 volumes NCLB buffer/g of wet cells (e.g., 200 ml NCLB buffer/100 g of cells). Cells can be stored at −80 °C.
- For the FreezerMill, use 1/3 volume NCLB buffer/g of cells (e.g., 33 ml NCLB buffer/100 g of cells). Drip the resuspended cells from a 25-ml pipette directly into liquid nitrogen (small droplets are formed). Can be stored at −80 °C.

5.3. Tip

YPD plates can be used initially but a selective medium must be used for the overnight culture.

5.4. Tip

YPD can be used for the larger volumes if tests do not indicate a selective loss of the plasmid(s) expressing the proteins to be purified.

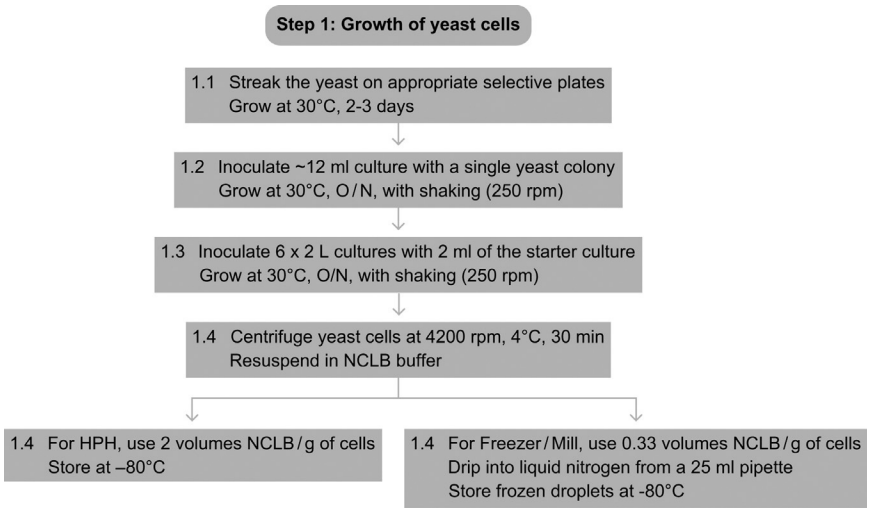


Figure 12.2 Flowchart of Step 1.

5.5. Info

In eIF3 purification, the yeast is streaked on an YPD plate and colonies from the YPD plate are used to inoculate an overnight culture of 50 ml SC-URA-LEU. Six 5-l baffled flasks, each containing 2 l YPD, are inoculated with 2 ml of overnight culture. After ~16 h of growth, the cells are collected (90 g of wet yeast cells were collected) and resuspended in 1/3 volume of NCLB buffer (30 ml) and dripped into liquid nitrogen and stored as droplets at −80 °C.

See Fig. 12.2 for the flowchart of Step 1.

6. STEP 2 LYSIS OF THE YEAST CELLS

6.1. Overview

The method used for breaking open the cells can be crucial in determining the amount and quality of the expressed protein obtained, especially if the protein has a tendency to be degraded.

6.2. Duration

2–5 h

2.1 Break open the yeast cells using either a HPH or a Freezer/Mill.

- Using the HPH, thaw the cells and filter through cheesecloth, and then pass the resuspended cells through the HPH three times at

25 000 psi. Proceed directly to Step 3.2. (Handle according to the vendor's instructions.)

- Using the Freezer/Mill, lyse the cells by carrying out two rounds in the liquid nitrogen grinder. The powder generated can be stored at −80 °C. (Handle according to the vendor's instructions.)

6.3. Info

In the eIF3 purification the Freezer/Mill was used.

7. STEP 3 PURIFICATION OF THE PROTEIN

7.1. Overview

The type of tag and protein will determine the purification protocol to be used. The method for purifying the His-tagged protein complex, eIF3, is described below. Fig. 12.3 shows the purified eIF3 on a SDS-PAGE gel (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)) stained with Coomassie blue (see Coomassie Blue Staining).

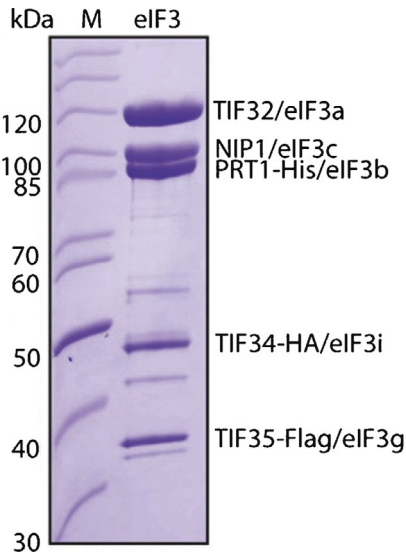


Figure 12.3 Purified eIF3, showing ~stoichiometric amounts of the five subunits.

7.2. Duration

2 days

3.1 If the Freezer/Mill was used to break open the cells, resuspend the cell powder in 200 ml of NCLB.

If the HPH was used to break open the cells proceed directly to Step 3.2.

3.2 Clarify the lysate by centrifugation at 12 000 rpm at 4 °C for 30 min.

3.3 During the centrifugation, set up and equilibrate a HisTrap nickel column in NCLB buffer on a FPLC or peristaltic pump.

3.4 Filter the supernatant from Step 3.2, first through a 5-μm filter and then a 0.8-μm filter and load the lysate onto the nickel column (2–3 ml min^{-1} flow rate).

3.5 Wash the nickel column with NCLB buffer until the UV absorbance at 280 nm reaches the baseline (5 ml min^{-1} flow rate).

3.6 Elute the bound protein with NCEB buffer (5 ml min^{-1} flow rate) and pool the entire eluate.

3.7 Concentrate the eluted protein using an Amicon Ultra (Millipore) concentrator (10 K MWCO) to a volume of 2 ml or less so that it can be applied onto a gel filtration column.

3.8 Apply the sample on a 120 ml Superose 12 gel filtration column that has been equilibrated in low salt buffer (0.5 ml min^{-1} flow rate) and collect 1 ml fractions during the run.

3.9 Analyze the fractions for the purified protein by SDS-PAGE (10% polyacrylamide gel) and Coomassie staining.

3.10 Pool appropriate fractions and store on ice at 4 °C overnight (End of day 1).

3.11 Apply the sample on a freshly prepared 10 ml phosphocellulose column equilibrated in low salt buffer by gravity flow.

3.12 Wash the column with 90 ml of low salt buffer.

3.13 Elute using a step gradient, four 5 ml fractions of 200 mM KCl buffer followed by four 5 ml fractions of 350 mM KCl buffer. Wash the column using 1 M KCl buffer.

3.14 Analyze the different eluted fractions by SDS-PAGE on a 10% polyacrylamide gel, followed by Coomassie staining. Pool the fractions containing stoichiometric amounts of the five eIF3 subunits.

3.15 Dialyze the sample into enzyme storage buffer.

Figure 12.4 Flowchart of Step 3.

3.16 Concentrate the sample to 200–500 μl (as in Step 3.7); measure the concentration and flash freeze in liquid nitrogen. The purified protein should be stored at −80 °C.

See Fig. 12.4 for the flowchart of Step 3.

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