An Effective Recombinant Protein Expression and Purification System in *Saccharomyces cerevisiae*

Running title: Recombinant Protein Production in Yeast

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Significance Statement

Expressing and purifying recombinant proteins are highly employed in biological and biomedical science. The traditional host organisms, especially *Escherichia coli*, for protein expression and purification, have certain limitations, due to lacking post-translational modification (PTM) systems and the insolubility of many recombinant proteins. The yeast protein production system has been an invaluable tool for the production of biopharmaceutical proteins, protein complexes, and the post-translationally modified proteins. Here we describe a detailed protein expression and purification protocol in *Saccharomyces cerevisiae* using galactose induction system. Budding yeast has rapid cell growth to reach a high density that creates a fast and easy eukaryotic protein production platform with high protein yield.

ABSTRACT

The expression and purification of recombinant proteins using the bacterial vector is a mature and preferred system to obtain folded and stable proteins. However, the functions of post-translational protein modifications, such as glycosylation or phosphorylation, could only be achieved using eukaryotic expression system. In addition, insolubility usually is another challenge of proteins expressed in *Escherichia coli*, such as certain intrinsically disordered proteins that are more prone to aggregation than folded proteins. The eukaryotic protein expression systems, including the human cell, baculovirus/insect cell, and yeast, have become the indispensable systems for the production of functional eukaryotic proteins. This unit describes a detailed protocol for performing cytosolic protein expression, protein purification, and protein characterization using budding yeast *Saccharomyces cerevisiae*. The introduced protein expression and purification system in yeast have advantages of low cost, high yield, high protein solubility, and the low requirement in expertise.

Keywords: Budding yeast • recombinant protein expression • protein purification • actin filament • post-translational modification

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INTRODUCTION

The functional study of proteins is central to our understanding of fundamental biological processes because every protein is different in their biochemical and biophysical properties. Both prokaryotic and eukaryotic protein expression systems are widely used for protein production. *Escherichia coli* expression system is usually considered initially for obtaining recombinant proteins over the higher organisms, though several challenges limit its application. In many cases, the targeted eukaryotic proteins that are not modified or appropriately folded may precipitate and form the inclusion bodies within *Escherichia coli*. Due to the demanding biochemical activities for the post-translational modified proteins, eukaryotic expression systems provides indispensable protein production platform, including the human, insects and yeast cells. Budding yeast *Saccharomyces cerevisiae* protein expression was characterized around thirty years ago (Herrmann et al., 1995; Holz, Hesse, Bolotina, Stahl, & Lang, 2002; Huo, Yu, Chen, & Li, 1993; Romanos, Scorer, & Clare, 1992). Several issues of plasmid instability, low protein yield, and inefficient cell grinding method limited the application of protein production in the yeast system. *Saccharomyces cerevisiae* could produce soluble recombinant proteins in the cytosol and introduce PTMs for eukaryotic proteins, which is an alternative approach for many problematic proteins that are not correctly expressed in *Escherichia coli*. Also, yeast expression system provides an excellent route for isolating and the functional studies of the protein complex. For example, rapid *in situ* purification of the protein complex and the binding partners have been successfully achieved in yeast before using tandem affinity purification (TAP) tagging (Puig et al., 2001). In this unit, we describe a step-by-step protocol for obtaining the recombined proteins from budding yeast. The central parts of the experimental procedure are presented in strategic planning.

STRATEGIC PLANNING

See Figure 1

BASIC PROTOCOL

SAMPLE PREPARATION FOR RECOMBINANT PROTEIN EXPRESSION AND PURIFICATION

Introductory paragraph

The described protocol uses a Gal1/10 promoter-based galactose-induction system for expressing the cytosolic recombinant protein in budding yeast. Cell grinding could be performed in the liquid nitrogen-based apparatus with a breaking efficiency of >99%. Simple immobilized metal affinity chromatography (IMAC) purification of polyhistidine-tagged protein is used, which is coupled to a following gel filtration chromatography if a further purification is required. The whole procedure of protein expression and purification is breakdown into following four main parts, plasmid construction, yeast transformation and protein induction, cell grinding, and fast protein liquid chromatography (FPLC) based protein purification.

Materials

*For the recipe, see **Reagents and Solutions**.*

Expression vector pYeastPro: *pGAL-ORF-3xStreptagII-9xHis* (modified from an *Escherichia coli* cloning vector pBR322, protein is expressed under the galactose promotor)

Transformation and protein production yeast strain YMY1032: *MATa ade2 leu2 his3 trp1 ura3 lys2::pGAL1:GAL4::LYS2 pep4::HIS3 bar1::hisG*

Transformation-competent *Escherichia coli*

LB plates containing 100 µg/ml ampicillin (see recipe)

LB medium containing 100 µg/ml ampicillin (see recipe)

YPD plate (see recipe)

YPD medium (see recipe)

Yeast transformation resuspension buffer (see recipe)

Yeast transformation buffer (see recipe)

Single-strain herring sperm carrier DNA (Promega, cat.no. D1815)

Synthetic minimal media (SMD) plate made with Dropout Medium (DO) lacking uracil (see recipe)

SMD with DO with 2% Raffinose but lacking leucine (see recipe)

30% Galactose, filter sterilized (MP-Bio, cat.no. 02101747)

10X YP medium (see recipe)

Glass beads 0.5 mm (Biospec, cat.no. 11079105)

18G 1 ½ TW needle

HEN buffer (see recipe)

HENN buffer (see recipe)

HisTrap Binding buffer (see recipe)

HisTrap Elution buffer (see recipe)

Gel filtration buffer (see recipe)

5X SDS protein loading Buffer (see recipe)

1X SDS-PAGE running buffer (see recipe)

GelCode™ Blue Stain Reagent (ThermoFisher, cat.no. 24592)

Liquid nitrogen

1.5-ml microcentrifuge tubes

1.5-ml screw-cap tubes

15- and 50-ml conical tubes

Shaking incubators (30°C and 37°C)

Spectrophotometer

Beckman JA25.5 and JLA9.1 rotors (or equivalents)

Analytical Balance

Weighing spoon

Sieve

Conical funnel

Special equipments

Beads beater: 6870 Freezer/Mill (SPEX SamplePrep, USA)

GE Healthcare Life Sciences FPLC purification system including:

GE Amersham AKTA Purifier 10

10 or 50 ml super-loop

5 ml HisTrap HP column (GE Healthcare, cat.no. G13/17-5248-01)

5 ml sample loop

HiLoad 16/600 Superdex 200 pg (GE Healthcare, cat.no. G13/28-9893-35)

1Subcloning of expression vector for the recombinant protein

1. Sub-clone the target DNA fragment into the pYeastPro vector for protein expression.

   The linearized vector is around 8.7kb, with a BamHI cut site in front of the ORF as well as PacI cut site after the ORF, various sub-cloning strategies can be chosen, such as double digestion with restriction enzymes or Gilson assembly.
2. Transform the ligated plasmids into the competent *E. coli* cells, select resistant transformants on LB plates containing ampicillin at 100 mg/ml after 12-16 hr at 37°C.
3. Inoculate 3-5 positive transformants into 5 ml LB medium containing ampicillin. Every single colony should be inoculated into one test tube and cultured with vigorous agitation at a speed of 250 rpm for 12-16 hr at 37°C.

4. Extract plasmids and verify the inserted DNA fragment by sequencing.

   Some simple verification methods could also be used to screen the positive inserts, such as colony PCR or digestion with restriction enzymes.

Transformants small-scale screening for protein expression

5. Inoculate the YMY1032 yeast stain from a glycerol stock onto a YPD plate at 30°C for 20-24 hr.

   *The YMY1032 yeast stain grows relatively slower than the wild-type strain, due to the deletion of protease protein and will turn pink after long time growth at 30°*C *after the adenine became depleted in the media.*

6. Inoculate 5-10 freshly grown colonies from the YPD plate into a test tube with 2 ml YPD medium, grow overnight at 30°C with vigorous agitation.
7. Measure the overnight culture density at OD_{600} by a Spectrophotometer, transfer the yeast culture into a 10-15 ml fresh YPD medium at a starting OD_{600} of 0.2.

   Yeast culture usually can reach up to 5-8 OD_{600}/ml after an overnight growth in YPD.

8. Shake the re-inoculated yeast culture with vigorous agitation at 30°C for 3-5 hr until OD_{600} reaches 0.5-0.8, centrifuge 2 min at 2,000 x *g* at room temperature in a 15 ml conical tube to collect yeast cells.

   It is recommended to measure the OD_{600} every two hours of growing, OD_{600} reaching 0.5-0.8 is the evidence of the healthy growth of the YMY1032 protease-deficient cells. A low-speed centrifugation is enough to pellet down the yeast cells, leaving a clear and transparent supernatant, which serves as evidence for free of bacterial contamination.

9. Wash the yeast cell pellets once with 2 ml sterilized ddH_2O, centrifuge at room temperature for 2 min at a speed of 2,000 x *g* to collect yeast.
10. Re-suspend the yeast cell pellets in a 1.5 ml Eppendorf tube with 0.1 ml yeast transformation resuspension buffer. Add 5 μl of single strain herring sperm carrier DNA (10 mg/ml), and 0.1 to 1 μg of the protein expression construct to the re-suspended yeast cell pellets.

    Increasing the DNA amount will result in a higher transformation efficiency. The volume of added DNA is not a crucial parameter, ideally up to 50 μl of the DNA can be added. The single-strain herring sperm carrier DNA needs to be boiled at 95°C for 10 min before the *first time usage, kept them in the -20°C fridge for storage and thaw on ice before every using.*

11. Add 700 µl of yeast transformation buffer and mix by vortexing.

    The yeast transformation buffer should be prepared freshly every time before using.

12. Incubate the cell mixture at 30°C for 30 min without shaking.
13. Vortex the cell mixture briefly and heat-shock at 42°C for 15-30 min.

    In most of the cases, 15 min is sufficient, but a longer heat shock time (up to 45 min) will possibly increase the transformation efficiency.

14. Centrifuge for 2 min at 2,000 x *g*, room temperature to collect yeast cell pellets and remove the supernatant.
15. Re-suspend the yeast cell pellets with 200 µl sterilized ddH_2O.
16. Spread the yeast cells on the *SMD DO* plate lacking uracil for selection. Incubate the plate at 30°C for 48 hr for autotrophic selection.
17. Inoculate 10-20 positive transformants into 5 ml *SM DO* media with 2% raffinose but lacking leucine, grow at 30°C with vigorous agitation in a shaking incubator until saturated.

    Another set of the colonies can be inoculated at the same time in a different test tube for small-scale protein expression test. It usually takes around 2 days for the culture to reach a saturation point (2-3 of OD_{600}) when the culture turns to be white.

18. Transfer the 5 ml culture to 30 ml *SM DO* media with 2% Raffinose but lacking leucine, allow a growing at 30°C with vigorous agitation until saturated, keep 15 ml culture in the 4°C fridge.

    10 ml of the 15 ml culture can be used as a control in the small-scale expression test (for step 20-32), the remaining 5 ml culture will be inoculated for a more substantial culture once positive results are obtained from the small-scale test (step 33).

    Usually, it takes around 36-48 hr to reach saturation.

19. Add 30% galactose stock to a final concentration of 2%, and add 10X YP medium to a final concentration of 1X YP into the remaining 20 ml culture (from step 18), grow at 30°C with vigorous agitation for 12-16 hr for protein induction.

    In addition to the YP nutrients, sufficient oxygen can also lead a better protein production, which could be achieved by using the flask with loose or vented caps.

20. Measure OD_{600} of the 15 ml culture prior to induction (from step 18) and after induction (from step 19). Collect yeast cells of a total OD_{600} of 15 for each sample by centrifuging 2 min at 2,000 x *g* at room temperature.

    The following steps (21-28) will be conducted for both samples side by side: 1) yeast culture before induction; 2) yeast culture after induction. The soluble protein fractions of the sample before and after induction will be compared to check for protein expression.

21. Wash yeast cells (before/after induction) once by re-suspending yeast cell pellets with 1 ml sterilized ddH_2O in a 1.5-ml screw-cap tube, centrifuge 2 min at 2,000 x g, room temperature.

    The cell lysing step (step23) requires the screw cap tube to sustain the high force of the bead-beater.

22. Apply 250 µl HEN buffer (with protease inhibitor) to re-suspend the yeast cell pellets, then add 100 µl 0.5 mm glass beads to the tube.

    The total volume of the cell culture, as well as the glass beads, should not exceed ½ of the total volume of the tube to ensure the efficient cell lysing.

23. Lyse the yeast cells by a Bead-beater with a power of 6,500 rpm for 30 sec, cool down the sample on ice for 1 min, and repeat twice.

    Make sure to cool down the tubes between each breaking cycle; this is to prevent sample accumulation at high temperature. Dry ice is a better alternative way of cooling. If a high power bead beater is not available, the lysing can be performed by a lower speed vortex mixers at 4 degree for 10-15 min.

24. Punch a hole at the bottom of the 1.5-ml screw-up tube with 18G 1 ½ TW needle, and place the tube on top of a 5 ml polystyrene round-bottom tube to collect the flow-through supernatant.
25. Centrifuge 2 min at 2,000 x *g*, 4°C to collect the flow through in 5 ml tube.
26. Wash beads twice by adding 200 µl of HENN buffer to the 1.5-ml screw-up the tube, centrifuge 2 min at 2,000 x *g*, 4°C to collect the flow-through in same 5 ml round-bottom tube.
27. Transfer the flow-through from the 5 ml polystyrene round-bottom tube to a new 1.5-ml Eppendorf tube, centrifuge 10 min at 14,000 x *g*, 4°C to remove the remaining cell debris.
28. Take out 40 µl of the supernatant, mix with 10 µl of the 5 X SDS proteins loading buffer in another 1.5 ml tube, boil the protein loading sample at 95°C for 10 min.
29. Prepare a 10% SDS-PAGE gel and load 10 µl of the sample from each: 1) Total cytosolic protein fraction before induction; 2) Total cytosolic protein fraction after induction.

To have a clear staining without overloading, a total protein lysate from an equivalent of 0.1-0.5 OD of cells is recommended to be loaded onto an SDS-PAGE for Coomassie blue staining. For example, yeast cells of a total OD_{600} *equal to 15 were collected in this protocol at step 20 and end-up to around 600 ul supernatant after lysis, resulting in a protein sample of 0.025OD of cell/ul. A loading of 10 ul gave a final equivalent of 0.25OD of cells.*

30. Run SDS-PAGE at a constant voltage of 120 V for 90 min. Stain the gel with 10 ml GelCode™ Blue Stain reagent at room temperature for 30 min.
31. Stop staining once clear bands are observed, then wash the gel with ddH_2O until background of the gel turns to be transparent.
32. Identify whether the target protein is expressed by comparing the samples before and after induction, see Figure 2.

    For the proteins with distinguishable size that can be identified by a Coomassie blue protein staining, a following large-scale protein expression and production could be performed directly. If not, a western-blot method can be used to detect the protein production by anti-HIS antibodies. For any protein with low yield, optimization is recommended before large-scale protein production, by either modifying protein with fusion tags or optimizing the induction condition, such as the culture or induction duration.

Large-scale cell culture for protein production

33. Transfer the remaining 5 ml culture (after showing a high yield of the target protein from the small-scale test) to 100 ml of raffinose-containing SM (synthetic drop-out medium without leucine, 2% *Raffinose*), grow at 30°C with vigorous agitation for 36-48 hr until saturated.
34. Scale-up the culture by transferring the 100 ml culture (from step 33) to 1.9 liters of fresh raffinose-containing SM in a 5-liter flask, with an additional culture for 36-48 hr at 30°C with vigorous agitation until saturated.
35. Add 160 ml 30% Galactose and 240 ml 10X YP medium to the 2 liters culture (from step 34), resulting in a medium with a final concentration of 2% galactose and 1X YP, grow for additional 12-16 hr with vigorous agitation at 30°C for protein production.

    Remember to leave a relatively loose capping on the culturing flask to provide sufficient oxygen for cell proliferation and protein production.

36. Collect yeast cell pellets by centrifugation at 6,000 x *g* for 5 min at 4°C using rotor JLA 9.1 in Beckman high-speed centrifuge, wash the yeast cell pellets once with 100 ml sterilized ddH_2O, and pellet down the yeast cells again with the same condition.

    Combine all the yeast cell pellets into one centrifuge tube during the washing step to facilitate following centrifugation steps.

37. Re-suspend the yeast cell pellets in 10 ml sterilized ddH_2O and transfer all re-suspended yeast cells to a new 50-ml conical tube, top up with sterilized ddH_2O to a final volume of 50 ml and vortex vigorously.
38. Collect the yeast cell pellets by centrifuging 15 min at 4,000 x *g*, 4°C.
39. Re-suspend the yeast cell pellets using sterilized ddH_2O of around 20% volume of cell pellets (e.g., 2 ml ddH_2O to 10 ml of pellets), then mix by pipetting up and down a few times with a 10 ml/25 ml pipette. Measure the cell mass on a weighing balance.
40. Freeze down the yeast cells into small frozen-pellets by dripping the resuspended cells slowly into a bucket of liquid nitrogen using a 25 ml Pipette.

    Carefully handle the liquid nitrogen with cry gloves, drip droplets slowly to prevent the formation of big frozen droplets cluster.

41. Label a new 50-ml conical tube, pre-cool it with liquid nitrogen. Collect frozen cell droplets using a pre-cooled mesh sieve and quickly transfer them into the labeled conical tube by passing through a pre-cooled plastic funnel, keep the tube in – 80°C freezer.

Cell grinding

42. Prepare liquid nitrogen to cool down the cryomilling machine and the grinding vial first, and transfer the frozen cell droplets into the grinding vial and cap it correctly.

    Carefully handle the liquid nitrogen with cryo-glove, the cryomilling machine requires around 5-liter liquid nitrogen to cool down, remember to top up the liquid nitrogen to the safety line after the cooling down step due to the fast evaporation. Choose the correct size of the grinding vial based on your sample mass and texture. The big size vial is suitable to hold 5-20 g of the cell pellets, which is used in this protocol.

43. Perform six cycles of a cell grinding that comprises 3 min of beating and a 1 min interval for each cycle. Pause the machine and take out the grinding vial after the 3rd cycle, shake it quickly

to prevent the cell packing at the corner of the vial. Top up liquid nitrogen to the safety line level at the meanwhile if it is necessary.

The sufficient cell breaking will result in the fine cell powder, increase the grinding cycle if it is necessary. Shaking once of the vial is critical in increasing the efficiency of cell breaking.

44. Transfer the cell powder from the grinding vial into a labeled pre-cooled 50-ml conical tube by quickly pouring all the cell powder through a pre-cooled conical funnel with the help of a pre-cooled metal weighing spoon. Keep the cell powder in –80°C deep freezer.

The powder can be kept in –80°C freezer up to one year for protein purification.

Alternatively, French Press technology, or cryomill grinder from Retsch, or steel blender 7011S from Waring can be used for grinding as previously described (Michelot & Drubin, 2014).

Protein purification: Affinity chromatography and gel filtration chromatography

45. Transfer 5-10 g of cell powder directly into a five-volumes of ice-cold HisTrap binding buffer on a weighing balance, and vortex thoroughly.

Transfer quickly to avoid thawing the remaining cell powders.

46. Centrifuge the re-suspended cell powder 30 min at 60,000 x *g*, 4°C with rotor JA 25.5, and transfer the supernatant carefully to a new 50-ml conical tube without disturbing the pellet, and filter the supernatant by a 0.22 μm pore size filter for the following purification. Keep 20 μl as "Original lysate before injection" sample for SDS-PAGE.

If the supernatant is turbid and hard to be filtered, repeat the centrifugation step.

47. Wash the affinity column by 5 column-volumes of degassed and filter-sterilized ddH$_2$O, follow by 5 column-volumes of the HisTrap binding buffer for equilibration while performing the centrifugation step (step 46).

In this protocol, a 5ml HisTrap column with a total binding capacity of up to 200 mg protein was used. The HisTrap column size can be chosen based on the availability.

48. Load the filtered protein sample into the injection loop with an appropriate size, connect the loop to a AKTAPurifier 10 FPLC machine, inject the sample into the column by a low flow rate to allow a sufficient binding of target protein to the HisTrap column, collect the flow-

through fraction, and keep 20 µl as “HisTrap binding flow-through” sample for SDS-PAGE examination.

49. Wash the HisTrap column by 10 column-volumes of the HisTrap binding buffer, keep 20 µl of the washing fraction as “Washing” sample for SDS-PAGE.

    The flow rate was set as 1 ml/min in this protocol, which did not exceed the column sustainable pressure limit (0.3MPa).

50. Elute protein by 10 column-volumes of mixed HisTrap binding and elution buffer that generate a linear gradient of imidazole (20–500 mM), followed by additional 5 column-volumes of HisTrap Elution buffer. Keep the same flow rate as the washing step (step 49) and collect 1 ml eluent per fraction.
51. Select the elution fractions based on the UV280 profile, take 20 µl from each selected fraction for SDS-PAGE.
52. Prepare 10% SDS-PAGE, mix samples of previous steps (46, 48, 49 and 51) with 5X SDS loading buffer, respectively, boil at 90°C for 10 min. Load 10 µl of each protein sample for SDS-PAGE running and staining, which are essentially the same as above small-scale test (step 30).
53. Stop the staining once the bright bands on the gel are observed, and wash the gel by ddH$_2$O until the gel background turns to transparent.
54. Combine the ideal elution fractions containing the target protein (Figure 3, Peak 2), proceed to gel filtration for further protein purification and buffer exchange.
55. Choose a suitable gel filtration column based on the difference of molecular weight between the target protein and the contamination proteins.

    In this protocol, a HiLoad® 16/600 Superdex® 200 pg column was used.

56. Wash the column with 1.5 column-volumes of filtered ddH$_2$O, followed by column equilibrium using 1.5 column-volumes of Gel filtration buffer.

    Monitor the column pressure by appropriate adjustment of flow rate. In this protocol, the flow rate was 1 ml/min that did not exceed the column pressure limit (0.5 MPa).

57. Concentrate down the sample volume to 5 ml by centricon if the combined elution protein fractions exceed 5 ml. Take out 20 µl as “Original Protein for Gel Filtration”.

    Choose the centricon with a specific cut-off size based on the molecular weight of the target protein. Here, a 50 kDa cut-off centricon was used for MBP-Bni1FH1COOH.

58. Load the concentrated HisTrap elution sample into the 5ml injection loop, connect the loop to the AKTAPurifier 10 FPLC machine, run gel filtration chromatography to separate proteins based on size difference by one column-volume of Gel filtration buffer, collect 2 ml per elution fraction.
59. Select the fractions for SDS-PAGE examination based on the predicted protein molecular weight and UV280 profile.

    Gel filtration column was pre-calibrated with protein standards as a reference for estimating elution volume of the target protein.
60. Prepare 10% SDS-PAGE, mix samples (from steps 57 and 59) with 5X SDS loading buffer, respectively, boil at 90°C for 10 min. Load 10 μl of protein sample for SDS-PAGE running and staining as described in step 30.
61. Destain SDS-PAGE gel as described in step 31.
62. Identify the elution fractions contain the pure target proteins on the gel, and combine all the pure fractions for following concentrating step, see Figure 4.
63. Concentrate the protein fractions by a centricon and measure the final protein concentration by either the NanoDrop Spectrophotometer or BCA Protein Assay. Aliquot the protein into small volume, fleshly freeze in liquid nitrogen, store in –80°C freezer.

REAGENTS AND SOLUTIONS

Use sterilized deionized, distilled water in all recipes and protocol steps.

For recipe of medium and solutions, see ***Table 1****;*

COMMENTARY

Background Information

The protocol described here is aimed at producing high-yield soluble eukaryotic proteins with posttranslational modification through a cytosol expression system of budding yeast. By carrying a leucine-deficient plasmid Pgal-ORF-StreptagII-6xHIS, the YMY1032 cell expresses weakly the functional LEU2, which requires a high-copy of plasmid when grown in leucine-dropout medium (Erhart & Hollenberg, 1983; Miao et al., 2016; Peng et al., 2015; St-Pierre et al., 2009). To reduce the intracellular proteolysis and to obtain a higher yield of the target protein, protease deficient strains are recommended for protein production (Gleeson, White, Meininger, & Komives, 1998; Tomimoto et al., 2013). The described yeast protein expression system in this unit has the following advantages. (1) Relatively inexpensive method compared to mammalian or insect expression system. (2) Ideal for the studies of eukaryotic proteins that require functional PTM. Protein PTMs of many eukaryotic proteins are crucial for their biochemical activities, including the protein-protein interaction, protein conformational changes, or activities in driving the specific cellular process (Haynes et al., 2006; Yansong Miao et al., 2013). (3) High protein solubility. Recombinant proteins prone to aggregate in bacteria-based systems is a significant challenge in most biochemical and biomedical research. Though refolding processes have been successfully applied to specific proteins, in many cases, the refolding steps are practically tricky and may affect the biochemical or biophysical characterization (Vincentelli et al., 2004). One particular example is the intrinsically disordered proteins (IDPs). As the network hubs, IDPs play essential regulatory roles in multiple biological processes, which occupy more than 30% of the eukaryotic proteome (Haynes et al., 2006). IDPs are heavily modified by posttranslational modification in the disordered region (Iakoucheva et al., 2004), especially the phosphorylation. Due to the conformational nature of dynamic and flexible, IDP tends to form heterologous protein aggregates (De Simone et al., 2012; Dunker, Brown, Lawson, Iakoucheva, & Obradovic, 2002). The yeast expression system is excellent in increasing the protein solubility as well as retaining the PTMs. (4) High-efficient cell breaking method and convenient cell powder storage. Low-efficient cell lysis was one of the limiting factors that hindered the development of protein production using yeast cytosolic protein expression system. Here, liquid nitrogen based-mechanical cell disruption achieves >99% cell breaking efficiency in ultra-low temperature and allows a direct storage of broken cell powder in -80°C for future purification without further cell grinding before each purification. So far, we found the broken yeast powers are stable for more than one year without a noticeable decrease in biochemical activities of the recombinant proteins. For each purification, 1-5 grams of yeast powder for protein purification is sufficient for most of the biochemical assays. (5) Another significant advantage of the described protein production system is its high compatibility for structural studies that require functional protein or protein complex with appropriate PTM in cryo-electron microscopic examination. However, not every protein of interest is expressed in *Saccharomyces cerevisiae* to high titers. The hetero-oligomers, hydrophobic regions, or toxic effects of the expressed proteins in yeast might lead to low protein yield.

Critical parameters and Troubleshooting

Small-scale screening of recombinant protein induction

For initiating a small-scale culture test, inoculate several colonies instead of a single colony into *SM DO* selection medium lacking leucine to allow fast cell growth. To maximize the yield of cell density and protein production, adding the galactose for induction until the raffinose culture is saturated. A supplement of proteinase inhibitors is critical to prevent protein degradation during purification.

Collection and cell grinding for large-scale culture

For scaling-up, the tested positive sample from small-scale test should be used directly to avoid potential sample-to-sample variation. For the cell breaking, a cryo-based grinding method is preferred to maintain the biochemical activities of the recombinant protein and enable a storage of cell lysate as the powder, which significantly saves the time for following protein purifications.

Protein purification

One major challenge of using yeast lysates for column chromatography is the viscous yeast cell lysates containing polysaccharide of the cell wall and membrane debris. As a result, a filtering step is crucial before injecting the total cell lysates into the purification column. Another way to decrease the cell lysates viscosity is to dilute the cell lysates with protein purification buffer, 1:5 dilution works well for most of our currently tested yeast protein purification. Due to the complex metabolic components, the yeast lysates appear to decrease the blue color of HisTrap column after several times of lysate loading. Usually, we perform a recharge of Ni^{2+} every five-injections to recover the protein binding capacity of the column. When purifying the IDPs or proteins with a high proportion of charged residues, a protein aggregation might occur. In such case, the high ionic strength of the buffer with 0.5-1M KCl or an additional 10% glycerol can be beneficial. Increase the use of cell powder amount for protein purification if the recombinant protein is at a relatively low expression level. Appropriate column chromatography needs to be explicitly chosen depends on the recombinant protein. For the proteins with high expression level and solubility, HisTrap coupled with gel filtration column chromatography usually work well for most of the cases.

Anticipated results (understanding results)

2 liters of yeast culture usually generates ~10-15 g of yeast powders. Depends on the expression level of the target protein, 0.5-2 mg protein in final would be expected for each gram of yeast powder. With eukaryotic PTM that is more close to the *in vivo* protein states, the purified protein could have a high biochemical activity. Here, we describe a particular application of this system to produce an FH1COOH region of yeast actin nucleation factor Bni1 (Y. Miao et al., 2013), which is fused with an N-terminal tag of maltose binding protein (Figure 5A). To test the activity of the purified Bni1 in nucleating actin filament, we performed pyrene-actin polymerization assay. A faster actin polymerization rate was observed in a protein concentration-dependent manner, as shown in Figure 5B.

Time Considerations

The construction of overexpression plasmids takes ~5-7 days. Transformation yeast strain and obtaining positive transformants need ~3 days. The small-scale tests for protein expression and solubility take ~3 days. Following large-scale culture for protein induction requires ~4-5 days. The cell grinding by the cryo-milling machine takes ~ 3 hr. The protein purification requires ~8-9 hr for affinity chromatography, another ~8-9 hr for gel-filtration chromatography, and finally ~1-3 hr for concentrating the recombinant protein.

ACKNOWLEDGEMENTS

YX, XH, and YSM wrote the manuscript. YX prepared the experimental reagent and performed the protein purification and activity test. This work was supported by Nanyang Technological University NAP start-up grant to YSM (M4081533), Academic Research Fund Tier 2 (MOE-2016-T2-1-005) and Tier 1 (RG38/17) to YSM from Ministry of Education in Singapore.

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FIGURE LEGENDS

Figure 1. Flowchart of experimental procedure.

Figure 2. Representative GelCode staining of SDS-PAGE gel for an expressed maltose binding protein (MBP)-Bni1FH1COOH. Lane 1: Total cytosolic protein before induction (from 0.25 OD of cells); Lane 2: Total cytosolic protein before induction (from 0.5 OD of cells); Lane 3: Total cytosolic protein after induction (from 0.5 OD of cells).

Figure 3. HisTrap affinity chromatography purification. A. UV280 profile of HisTrap chromatography purification. B. 10% SDS-PAGE gel of the HisTrap chromatography purification result. Lane 1: Original lysate before injection; Lane 2: Flow-through from HisTrap column chromatography; Lane 3: Samples from washing step; Elution Peak1: Little target protein present but with high contamination proteins; Elution Peak 2: Target recombinant protein used for further gel filtration column chromatography. Star indicates the targeted protein MBP-Bni1FH1COOH.

Figure 4. Gel filtration chromatography purification. A. UV280 profile of the HiLoad® 16/600 Superdex® 200 pg column chromatography purification. Peak S2 corresponding to the predicted molecular weight of target protein, where Peak S1 indicates the oligomerized proteins. B. The SDS-PAGE gel to check protein fractions from gel filtration chromatography. The fractions from peak S2 were collected and concentrated for further biochemical assays.

Figure 5. Biochemical activity examination of purified protein. A. 10% SDS-PAGE gel of purified MBP-Bni1FH1COOH. B. Pyrene actin polymerization assay in the presence of 2μM of G-actin and MBP-Bni1FH1COOH at the indicated concentration.

Table 1. Recipe of medium and solutions

Name and quantity	Recipe	Storage
LB plates containing 100ug/ml ampicillin (500 ml)	1% w/v tryptone 0.5% w/v yeast extract 1% w/v NaCl 2% w/v Agar 100ug/ml ampicillin	Store 2-3 months at 4°C
LB medium containing 100ug/ml ampicillin (500 ml)	1% w/v tryptone 0.5% w/v yeast extract 1% w/v NaCl 100ug/ml ampicillin	Store 2-3 months at 4°C
YPD plate (500 ml)	1% w/v Yeast extract 2% w/v Peptone 2% w/v Glucose 2% w/v Agar	Store 2-3 months at 4°C
YPD medium (1 liter)	1% w/v Yeast extract 2% w/v Peptone 2% w/v Glucose	Store 2-3 months at room temperature
Yeast transformation resuspension buffer (100 ml)	100 mM Lithium acetate 10 mM Tris-HCl pH 8.0 1 mM EDTA pH 8.0	Store 6-12 months at room temperature
Yeast transformation buffer (2 ml)	40% w/v 3300 PEG (Freshly prepared from 50% stock) 100 mM Lithium acetate 10 mM Tris-HCl pH 8.0	Freshly prepared before every time using

	1 mM EDTA pH 8.0	
SMD DO Ura3 plate (500 ml)	6.7 g/L Bacto-yeast nitrogen base without amino acid 20 mg/L Adenine 20 mg/L L-Tryptophan 20 mg/L L-Histidine 20 mg/L L-methionine 30 mg/L L-Leucine 30 mg/L L-Lysine 2% w/v Glucose 2% w/v Agar	Store 2-3 months at 4°C
SM DO Leu 2% Raffinose medium (3 liter)	6.7 g/L Bacto-yeast nitrogen base without amino acid 20 mg/L Adenine 20 mg/L L-Tryptophan 20 mg/L L-Histidine 20 mg/L L-methionine 20 mg/L Uracil 30 mg/L L-Lysine 2% w/v Raffinose	Store 2-3 months at room temperature
10X YP medium (400 ml)	10% w/v Yeast extract 20% w/v Peptone	Store 2-3 months at room temperature
HEN buffer (with phosphatase inhibitors and protease inhibitor, 50 ml)	50 mM HEPES pH 8.0 1 mM EDTA pH 8.0 150 mM NaCl 1.6 µl/ml Protease inhibitor cocktail (Bio world) 1 mM Phenylmethane Sulfonyl Fluoride (PMSF)	Freshly prepared before every time using
HENN buffer (with phosphatase inhibitors and protease inhibitor, 50ml)	50 mM HEPES pH 8.0 1 mM EDTA pH 8.0 150 mM NaCl 0.5% v/v NP-40 1.6 µl/ml Protease inhibitor cocktail 1 mM PMSF	Freshly prepared before every time using
5X SDS protein loading buffer (1 ml)	10% w/v Sodium Dodecyl Sulfate (SDS) 10 mM Beta mercaptoethanol 200 mM Tris-HCl pH 6.8 20% v/v Glycerol 0.05% w/v Bromophenol blue	Store 6-12 months at -20°C
1X Running Buffer (5 liter)	25 mM Tris-HCl pH 8.8 200 mM Glycine 0.1% w/v SDS	Store 6-12 months at room temperature
HisTrap binding buffer (1 liter)	50 mM Tris-HCl pH 8.0 500 mM NaCl 20 mM Imidazole 1.6 µl/ml Protease inhibitor cocktail 1 mM PMSF	Freshly prepared before every time using
HisTrap elution buffer (500 ml)	50 mM Tris-HCl pH 8.0 500 mM NaCl 500 mM Imidazole	Store 2-3 months at 4°C
Gel filtration buffer (500 ml)	50 mM Tris-HCl pH 8.0 500 mM NaCl	Store 2-3 months at 4°C

Figure1

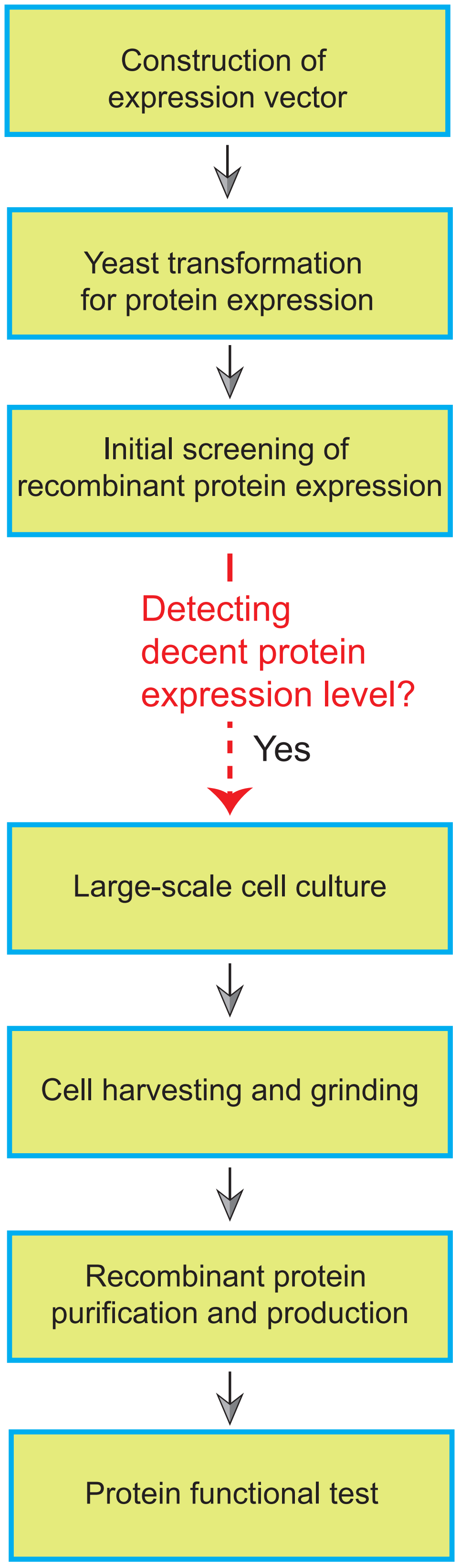

Construction of expression vector
Yeast transformation for protein expression
Initial screening of recombinant protein expression
Detecting decent protein expression level?
Yes
Large-scale cell culture
Cell harvesting and grinding
Recombinant protein purification and production
Protein functional test

Figure 2

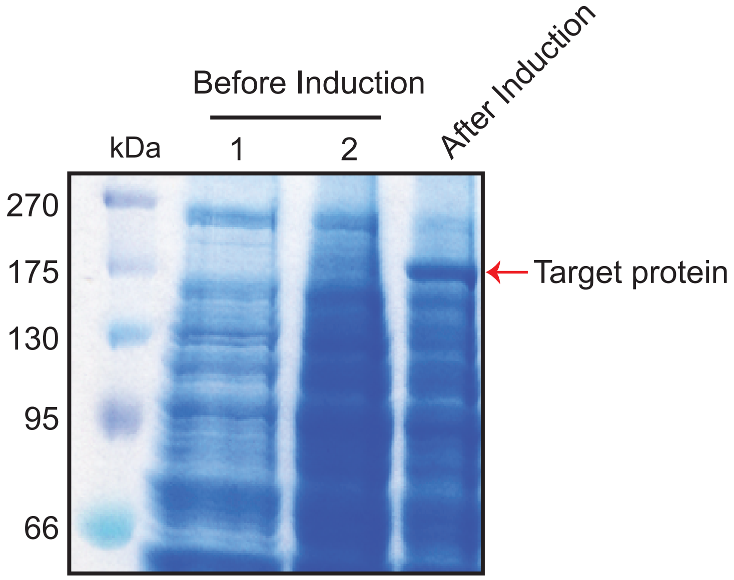

Before Induction
After Induction
kDa
1
2
270
175
130
95
66
Target protein

Figure 3

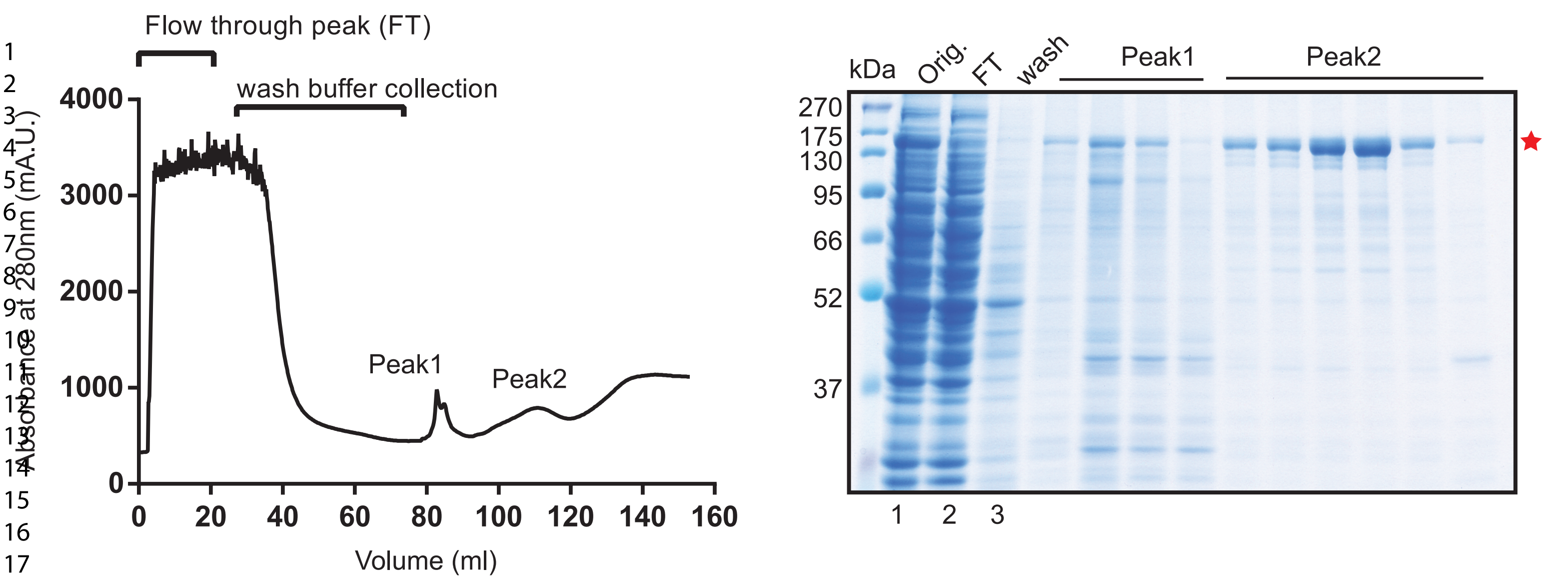

A.
Flow through peak (FT)
wash buffer collection
4000
3000
2000
1000
0
Absorbance at 280nm (mA.U.)
Peak1
Peak2
0
20
40
60
80
100
120
140
160
Volume (ml)
B.
kDa
Orig.
FT
wash
Peak1
Peak2
270
175
130
95
66
52
37
★
1
2
3

Figure 4

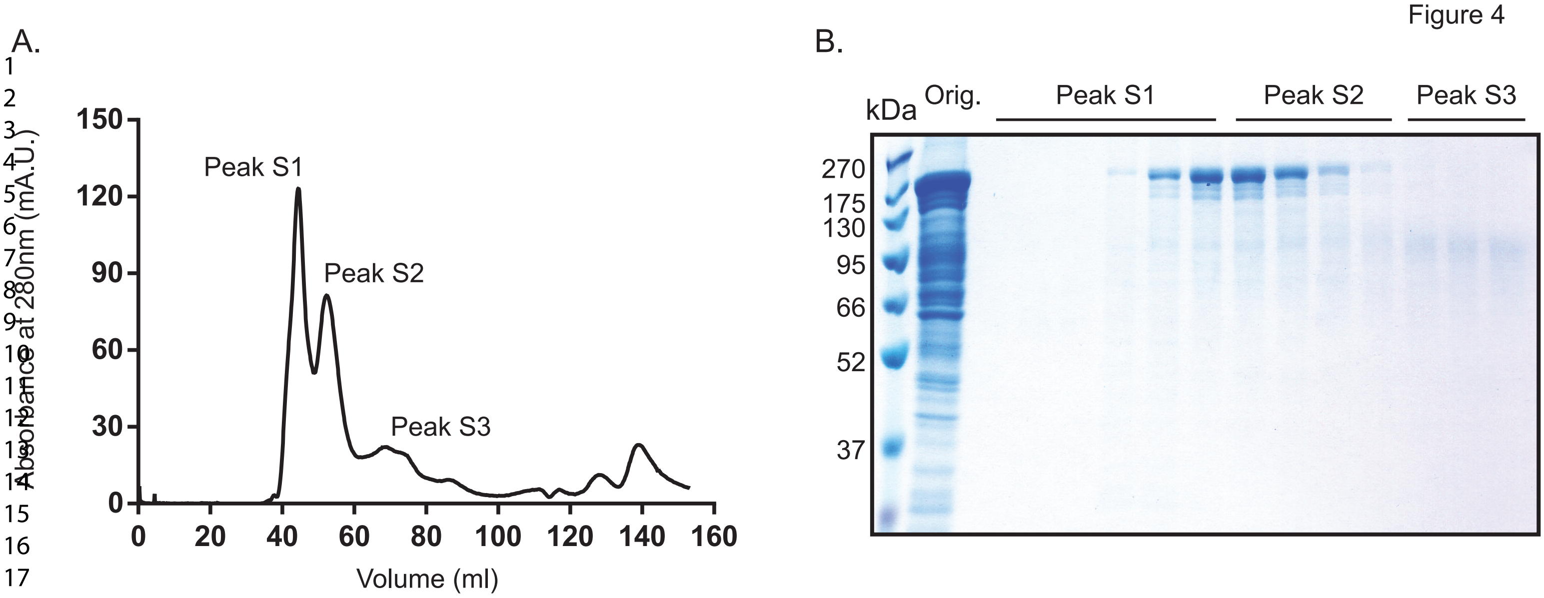
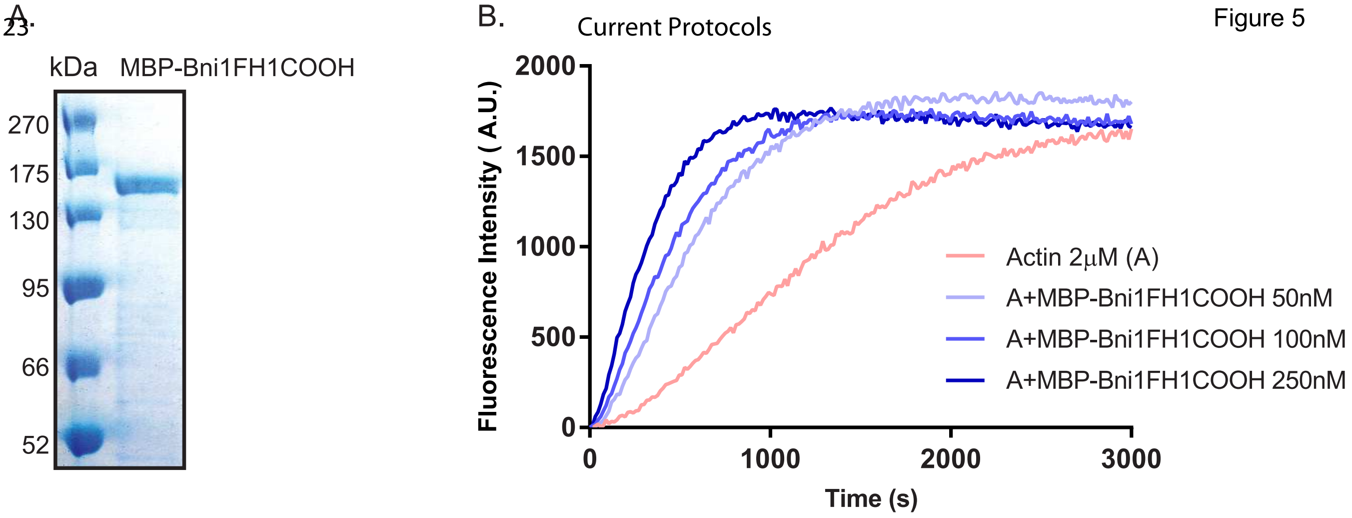

A.
Absorbance at 280nm (mA.U.)
150
120
90
60
30
0
Peak S1
Peak S2
Peak S3
0
20
40
60
80
100
120
140
160
Volume (ml)
B.
kDa
Orig.
Peak S1
Peak S2
Peak S3
270
175
130
95
66
52
37

Figure 5


A.
kDa
MBP-Bni1FH1COOH
270
175
130
95
66
52
B.
Fluorescence Intensity (A.U.)
2000
1500
1000
500
0
0
1000
2000
3000
Time (s)
Actin 2μM (A)
A+MBP-Bni1FH1COOH 50nM
A+MBP-Bni1FH1COOH 100nM
A+MBP-Bni1FH1COOH 250nM