# Single protein production (SPP) system in *Escherichia coli*

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**Here, we provide a detailed protocol for the single protein production (SPP) system, which is designed to produce only a single protein of interest in living *Escherichia coli* cells. Induction of MazF, an mRNA interferase that cleaves RNA at ACA nucleotide sequences, results in complete cell growth arrest. However, if mRNA encoding a protein of interest is engineered to be devoid of ACA base triplets and is induced at 15 °C using pCold vectors in MazF-expressing cells, only the protein from this mRNA is produced at a yield of 20–30% of total cellular protein; other cellular protein synthesis is almost completely absent. In theory, any protein can be produced by the SPP system. Protein yields are typically unaffected even if the culture is condensed up to 40-fold, reducing the cost of protein production by up to 97.5%. The SPP system has a number of key features important for protein production, including high-yield and prolonged production of isotope-labeled protein at a very high signal-to-noise ratio. The procedure can be completed in 7 d after cloning of an ACA-less target gene into the expression system.**



## INTRODUCTION

MazF is a toxin encoded by the *Escherichia coli* chromosome and is assumed to play an important role in stress adaptation[1–4]. It functions as an mRNA interferase, which specifically cleaves cellular mRNAs at ACA nucleotide sequences[5,6]. Its induction in *E. coli* causes a novel physiological state called 'quasi-dormancy,' under which cells are fully metabolically active and capable of synthesizing protein even in the total absence of cell growth[5].

Since MazF induction in *E. coli* causes degradation of almost all cellular mRNAs, cellular protein synthesis is dramatically inhibited, resulting in complete cell growth arrest. However, an mRNA in which all ACA sequences are altered to other sequences that are no longer cleavable by MazF can be very efficiently translated in cells overproducing MazF. Thus, only the protein from this mRNA is produced at a high yield in the absence of the production of any other cellular protein. Since any ACA sequence in an open reading frame (ORF) can be substituted with non-MazF-cleavable sequences without altering the original amino acid sequence (see **Fig. 1**), any protein may be produced by the single protein production (SPP) system. The SPP system has been previously validated; here, we provide a step-by-step protocol for implementing SPP, on the basis of published methods[5,7].

### Advantages of the SPP system

The unique, novel features of the SPP system are listed as follows:

**High signal-to-noise ratio.** Since the protein of interest is produced virtually in the absence of background cellular protein synthesis, labeling of this protein with isotopes such as $^{15}N$ and $^{13}C$ can be achieved efficiently with a very high signal-to-noise ratio[5,7]. More than 90% of the isotope is incorporated into the target protein in the SPP system. Therefore, NMR structural studies of the protein may be carried out without purification, as previously shown with pCold vectors[8].

This aspect is particularly important for structural studies of membrane proteins, as their purification is a major hurdle in their structural studies. Furthermore, the high signal-to-noise ratio may permit NMR structure studies of a protein of interest even inside the cell (in-cell NMR[9,10]).

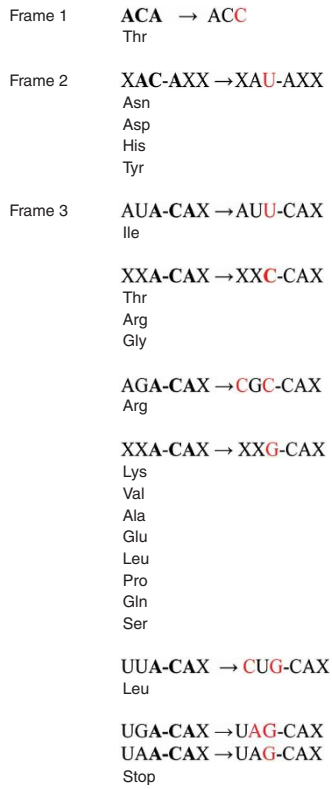


**Figure 1 |** Alteration of ACA sequences to non-MazF-cleavable sequences without changing amino acid sequences. ACA sequences are shown in bold. Altered codons are optimized for usage in *Escherichia coli*. Altered bases are in red. X represents a nucleotide, which is variable according to the amino acid code for the amino acid listed. X should not be changed when ACA sequences are altered.

**Efficiency of protein production.** Since, with the SPP system a protein of interest can be exclusively produced in living cells in the absence of other cellular protein production, cells are indeed converted into bioreactors for SPP. The level of protein production may be as high as 20–30% of total cellular proteins[5,7]. Notably, this level of protein production is not affected by incorporation of toxic amino acid analogs such as selenomethionine and fluorophenylalanine in the medium[7]. In the absence of active cellular protein synthesis, these analogs cannot be incorporated into cellular proteins, thereby minimizing their toxic effect.

**Cost-effectiveness.** Since the SPP system allows production of a protein of interest in the absence of cell growth, a cell culture can be condensed up to 40 times at the stage of isotope labeling or incorporation of amino acid analogs[7]. Therefore, a 1-l culture can be condensed to a 25-ml culture, which substantially reduces the amount of expensive materials such as amino acid analogs, $D_2O$, deuterated $^{13}C$-glucose, and $^{15}N$ $^{13}C$ double-labeled amino acids. This condensed SPP (cSPP) system consequently reduces the cost of protein production to 2.5% without affecting the protein yield. With the cSPP system, target protein can be produced at a level of up to 1.5 mg $ml^{-1}$ culture.

## Applications of the SPP system

The SPP system is run at low temperature as it uses cold-shock vectors. Therefore, it possesses all the advantages associated with cold-shock protein expression, such as better protein folding, and consequently, better protein solubility. Proteins expressed with other vectors may also be expressed in the SPP system. Notably, some toxic proteins that cannot be expressed in other systems may be expressed in the SPP system because in this sytem, cell growth is not required.

The application of the SPP system to membrane proteins is particularly attractive, since isotope-labeling of membrane proteins can be carried out with a very high signal-to-noise ratio. Thus, NMR structural study of membrane proteins may be carried out without purifying them to homogeneity.

## Limitations of the SPP system

Unlike other expression systems, the gene of interest has to be engineered to be devoid of ACA sequences. However, this seemingly major hurdle is not a serious problem anymore, due to the recent availability of affordable commercial gene synthesis.

The expression levels of proteins in the SPP system may widely vary due to their stability and toxicity. As a result, the optimum incubation period for the maximum production of a target protein may be different from protein to protein. Therefore, for each protein, conditions for optimum expression have to be determined by a small-scale pilot experiment. When the cSPP system is used, the maximum level of culture condensation without affecting the final protein yield may also be different from protein to protein. Therefore, one should empirically establish the best culture condensation condition for each protein by a small-scale pilot experiment to compare the protein yield of noncondensed culture with those of 10-, 20-, 30-, 40- and 50-fold condensed cultures.

## Comparison of the SPP system to other protein production strategies

There are two other strategies by which one can produce a protein of interest at a low background protein synthesis[11] as the following description.

**Cell-free systems.** Recently, cell-free systems using *E. coli*[12,13] and wheat germ[14–16] have become available for large-scale protein production. These cell-free systems also allow SPP as they use mRNA for a specific protein. However, the major drawback of the cell-free systems is that the systems by themselves are quite expensive. In addition, all 20 amino acids and many other factors, including an energy production system (ATP and GTP), have to be added to the reaction. For NMR structural study, all the amino acids have to be isotope-labeled, which substantially increases the cost of protein production.

In contrast, the SPP system uses a defined medium (M9 medium, see REAGENT SETUP) consisting of only $NH_4Cl$, glucose and phosphate buffer. The MazF-induced cells in the quasi-dormant state are fully metabolically active in the production of ATP, amino acids and nucleotides and retain their capacity for mRNA and protein synthesis[5].

**Inhibition of *E. coli* RNA polymerase by rifampicin.** With use of T7 vector systems, background cellular protein synthesis can be suppressed by the addition of rifampicin, since this antibiotic specifically inhibits *E. coli* RNA polymerase[17]. Therefore, SPP may be theoretically achieved with the use of T7 expression system in the presence of rifampicin. However, previous attempts to label a protein of interest with $^{15}N$ for in-cell NMR by this method did not improve the quality of HSQC spectra over those obtained from control experiments in the absence of rifampicin[18].

## Critical step in the SPP system: cloning of ACA-less genes

The most critical step in the SPP system is the preparation of an ACA-less gene for the protein of interest. If the gene contains only a few ACA sequences, each of these can be altered to a non-MazF-cleavable sequence by oligonucleotide-directed site-specific mutagenesis. However, if the gene is large, many ACA sequences are expected (on average, there is one ACA sequence per 64 bases). Therefore, site-directed mutagenesis to remove ACA sequences from this gene would be cumbersome and time consuming.

The problem can be circumvented by chemically synthesizing the entire gene. This is now economically feasible due to the development of new technology, which enables whole gene synthesis at an extremely low cost[19]. This will allow not only the elimination of all ACA sequences, but also importantly, adjustments to the codon usage to ensure optimum expression in *E. coli*.

## Availability of SPP system components

The SPP system is carried out by coexpression of *mazF* and an ACA-less gene that encodes the target protein. In theory, any expression vector can be used for the SPP system. However, there are some critical points.

- The *mazF* gene should be cloned into a low copy number vector, which regulates its expression very tightly. This will avoid complications arising from very high or untimely exertion of MazF toxicity.
- As MazF cleaves mRNAs at ACA sequences, the vectors for expressing protein of interest should not contain any ACA sequences in transcribed regions; thus, the 5′-and 3′-untranslated regions should be ACA-less in addition to the coding region used for protein fusion.
- Choice of expression vectors for a target protein is quite empirical as a particular vector may work better for that protein than others[8]. Therefore, it may be recommended to try more than one expression vector in initial trials of the SPP system. In this protocol, we use pCold plasmids.

### Future challenges

While the *E. coli* SPP system is expected to be widely used for a variety of purposes, the SPP systems in higher organisms such as yeast and mammalian cells may also be highly desirable for functional and structural studies of eukaryotic proteins as many of these may require post-translational modifications. Currently, these systems are being developed in our laboratory.

## MATERIALS

### REAGENTS

- pCold vectors (see REAGENT SETUP) [pColdI(SP-4), GenBank accession number AB248600; pColdII(SP-4), GenBank accession number AB248601; pColdIII(SP-4), GenBank accession number AB248602; pColdIV(SP-4), GenBank accession number AB248603]
  *Note:* All plasmids are available from TaKaRa Bio. Inc (cat. no. 3366-3370).
  ▲ CRITICAL Other expression systems, such as pET vectors, may be used for the SPP system if the expression of a particular target protein is better than with pCold vectors. However, in this case, all ACA sequences in the entire vector-derived transcript have to be altered to non-MazF-cleavable sequences without changing amino acid sequence of the protein.
- pMazF (see REAGENT SETUP)
- *E. coli* strains (see REAGENT SETUP)
- M9 medium (see REAGENT SETUP)
- M9-CAA medium (see REAGENT SETUP)
- M9-CAA agar plates (see REAGENT SETUP)
- Antibiotics (chloramphenicol, ampicillin)
- Phosphate buffer
- 10× M9 salts (see REAGENT SETUP)
- Isopropyl-β-D-thiogalactopyranoside (IPTG)
- ACA-less genes (Codon Devices)

### EQUIPMENT

- Toothpicks
- Petri dishes, 100 × 15 mm
- Shaker incubator, 37 °C (set to approximately 150 r.p.m.)
- Shaker incubator, 15 °C (set to approximately 150 r.p.m.) ▲ CRITICAL The temperature does not have to be exactly 15 °C. However, higher temperature (37 °C) causes poor expression because the toxicity of MazF seems to be too strong.

▲ CRITICAL All the equipment used for growing cells should be sterilized.

### REAGENT SETUP

**pCold vectors** With pCold vectors, a target protein can be expressed with a high signal-to-noise ratio at low temperature (15 °C), since the target gene is cloned under the *cspA* (major cold-shock protein) promoter. The expression of the target gene can be regulated by the addition of IPTG, since pCold vectors contain *lac* operator. pColdI, II and III vectors contain a translation-enhancing element (TEE) resulting in five extra residues at the N-terminal end of a protein. Using pColdI and II, attach a $(His)_6$ tag after the TEE. pColdI also has the recognition site for Factor Xa for cleaving the $(His)_6$ tag after purification. With pColdIV, the protein of interest is produced only from the initiation Met residue (**Fig. 2**).

***E. coli* strains** Most *E. coli* strains can be used as a host for the SPP system, including *E. coli* BL21(DE3), BL21 and W3110 strains, unless they carry chloramphenicol- or ampicillin-resistant markers, since pCold vectors carry the ampicillin marker and pMazF carries the chloramphenicol marker. In our laboratory, BL21 or BL21(DE3) is used for protein expression (Step 6 onward) and DH5α is used for plasmid construction (up to Step 5).

**pMazF** In this plasmid, the *mazF* gene is cloned into a low copy number plasmid, pACYC under a *lac* promoter, so that the *mazF* gene is inducible with 1 mM IPTG. This plasmid contains the chloramphenicol-resistance gene.

**M9 medium (per liter)** After autoclaving 900 ml water, the following materials are added: 100 ml 10× M9 salts, 1.0 ml 1 M $MgSO_4$, 0.1 ml 1 M $CaCl_2$, 10.0 ml 40% (wt/vol) glucose, 4.0 ml 0.5 mg $ml^{-1}$ vitamin $B_1$.

**10× M9 salts (per 100 ml)** 12.8 g $Na_2HPO_4 \bullet 7H_2O$ (6.8 g $Na_2HPO_4$), 3.0 g $KH_2PO_4$, 0.5 g NaCl, 1.0 g $NH_4Cl$ (pH should be adjusted to approximately 7.4).

**M9-CAA medium (per liter)** To M9 medium prepared earlier, add 10 ml 20% (wt/vol) casamino acids (CAA).

**M9-CAA agar plates (per liter)** Autoclave 15 g agar with 900 ml water and mix well. After adding materials required for M9-CAA medium, mix again and pour approximately 25 ml into each Petri dish.

## PROCEDURE

### Creation of ACA-less genes ● TIMING 2–15 d

**1|** Locate all ACA nucleotide sequences in the ORF of the gene to be expressed. This might be done by importing the sequence into Word and subsequently searching the sequence using the 'find' tool.

**2|** Use **Figure 1** to determine the appropriate base substitutions to replace all ACA nucleotide sequences while retaining the amino acid sequence of the encoded protein.

**3|** Introduce the required base changes to the DNA template. Appropriate restriction enzyme sites may be added at this stage to aid subsequent cloning steps. If only a small number of ACA sites needs to be removed, any standard oligonucleotide-directed site-specific mutagenesis method may be the most efficient approach (http://www.stratagene.com/manuals/200518.pdf). However, if large numbers of ACA sites need to be replaced, it is usually more efficient to chemically synthesize the ORF to incorporate the changes. It may be appropriate to have the remainder of the gene altered to take into account the codon bias of *E. coli* to increase expression levels of the protein. Any

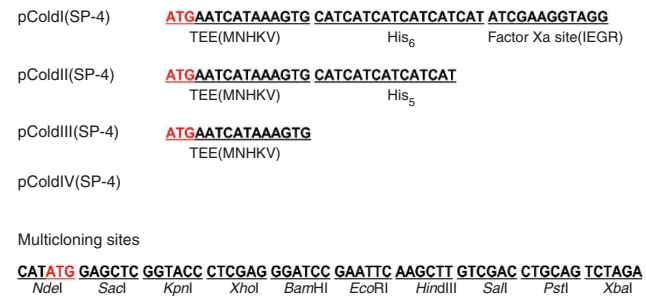


**Figure 2** | Multicloning sites in pCold(SP-4) vectors. These plasmids are derivatives of cold-shock high expression vectors, pColdI–IV[8], in which all ACA sequences from the 5′- and 3′-untranslated regions are altered to non-MazF-cleavable sequences. They are available from TaKaRa Bio. Inc. For each vector, the sequence shown is followed by the multicloning sites described at the bottom of the figure. With pColdIV(SP-4), an ACA-less gene is cloned at the *Nde*I site of the multicloning sites so that extra amino acid residues are not added at the N-terminal end of the protein except for the Met residue used for translation initiation. pColdI(SP-4) adds 17 residues, MNHKVHHHHHHIEGR↓HM, where an arrow indicates the Factor Xa cleavage site. pColdII(SP-4) adds 12 residues, MNHKVHHHHHHM, and pColdIII(SP-4) adds 7 residues, MNHKVHM, which corresponds to the translation enhancing element in the *cspA* mRNA[8]. The initiation codons are shown in red. ATG in the multicloning sites is used as the initiation codon only in pColdIV(SP-4).

synthetic methods can be used for gene synthesis; in our laboratory, ACA-less genes are synthesized by Codon Devices (http://www.codondevices.com).

**Cloning the ACA-less genes into the pCold vector ● TIMING 2–5 d**

**4|** Clone the ACA-less ORF obtained from Step 3 into one or more of the four pCold(SP-4) vectors (**Fig. 2**). Start by setting up individual restriction digests for the ACA-less gene DNA (insert DNA) and the appropriate vector(s) by using suitable restriction enzymes. Incubate as indicated in the manufacturer's instruction.

▲ **CRITICAL STEP** Any expression vector can be used, however, in this protocol, we have described an example using pCold vectors, which we have successfully used to avoid complications arising due to incompatibility of antibiotics or inducers. Any standard cloning method can be used. Recent technology development allows cloning of any genes into any vectors without restriction enzyme digestion and ligation (http://www.clontech.com/images/pt/PT3941-1.pdf). In this protocol, we describe a cloning method using restriction enzymes and ligase.

**5|** Purify the restriction-digested insert and the vector DNA by gel electrophoresis followed by gel extraction.

**6|** Set up the ligation reaction using the purified insert and vector DNA (the ACA-less gene and pCold vectors), as described in the instructions provided with the ligase.

**7|** Prepare competent cells and transform with the ligation reaction. Plate transformed cells on selective media and incubate overnight at 37 °C. The Inoue method is used in our laboratory[20] to prepare and transform competent cells; however, any method can be used.

▲ **CRITICAL STEP** Include a positive control (undigested vector DNA) to determine the competency of cells and a negative control (digested, unligated vector DNA or ligated vector DNA without insert) to determine the ligation efficiency.

**8|** Next day, screen for positive clones by colony polymerase chain reaction (PCR) using vector-specific primers or by other standard methods. Select the positive candidates according to the size of the PCR fragment compared to the undigested vector DNA transformed in Step 7. The PCR fragment from positive colonies is larger than that from the undigested vector DNA by the size of the gene insert.

▲ **CRITICAL STEP** Remember to restreak colonies used for PCR on fresh plates and grow at 37 °C overnight. These will be needed for subsequent isolation of DNA from positive clones.

▲ **CRITICAL STEP** DNA from candidate clones should be fully sequenced to confirm that they contain a perfect, unmutated copy of the ACA-less insert.

**9|** Prepare plasmid DNA for the expression plasmid selected in Step 8 for transformation. Any DNA preparation methods can be used; alkaline lysis with SDS is used in our laboratory[21]. For each transformation, 10 ng DNA is sufficient.

■ **PAUSE POINT** Plasmid DNA can be stored at −20 °C until required.

**Preparation of cells for expressing protein ● TIMING 5–7 d**

**10|** Pick a single colony of an appropriate host *E. coli* strain from the plate and grow in M9-CAA medium. Use this culture to prepare competent cells for transforming with pMazF, as in Step 7. The Inoue method is used in our laboratory[20] to prepare and transform competent cells, however, any method can be used.

■ **PAUSE POINT** Competent cells can be stored at −80 °C for several months.

**11|** Transform 50 µl competent cells with 10 ng pMazF and plate on M9-CAA agar plates containing 25 µg ml$^{-1}$ chloramphenicol. Incubate selective plates overnight at 37 °C. Only cells transformed with pMazF will form colonies on selective plates.

▲ **CRITICAL STEP** All transformation procedures have to be carried out using M9-CAA plates. LB medium should not be used as it contains contaminating *lac* inducers, which induce the *mazF* gene. This will consequently result in mutations in the *mazF* gene to reduce or eliminate the toxic effect of MazF. Therefore, for liquid cultures, M9-CAA medium should be used.

**12|** Next day, pick a single colony from the plate and grow in M9-CAA medium containing 25 µg ml$^{-1}$ chloramphenicol. Use this culture to prepare pMazF-containing competent cells, as in Step 7.

■ **PAUSE POINT** Competent cells can be stored at −80 °C for several months.

**13|** Transform 50 µl pMazF-containing competent cells prepared in Step 12 with 10 ng the expression plasmid DNA prepared in Step 9. Plate transformed cells on M9-CAA agar plates containing 25 µg ml$^{-1}$ chloramphenicol and 100 µg ml$^{-1}$ ampicillin to select the cells transformed with expression plasmid. Incubate the plates overnight at 37 °C. Only cells containing both plasmids will form colonies on selective plates.

▲ **CRITICAL STEP** All transformation procedures have to be carried out using M9-CAA plates. LB medium should not be used as it contains contaminating *lac* inducers, which may induce the *mazF* gene. This will consequently result in mutations in the gene of interest to counteract the toxic effect of MazF. Therefore, for liquid cultures, M9 or M9-CAA medium should be used.

**Induction of protein expression ● TIMING 2 d and reaction continues for 1 week**

**14|** Depending on the purpose of the experiments, three alternative methods can be used to induce protein expression. Option A should be used for protein production (not for specific labeling), option B should be used for incorporation of selenomethionine or other amino acid analogs and option C should be used for incorporation of isotopes ($^{15}N$ and $^{13}C$) or isotope-labeled amino acid analogs.

**? TROUBLESHOOTING**

**(A) Protein production**

(i) Pick a single colony (containing pMazF and the expression plasmid), using a toothpick, from freshly plated transformed cells in Step 13 and grow overnight in 50 ml M9-CAA medium containing 25 μg $ml^{-1}$ chloramphenicol and 100 μg $ml^{-1}$ ampicillin at 37 °C on a shaker (at approximately 150 r.p.m.).

(ii) Add the overnight culture to 1 l M9-CAA medium containing 25 μg $ml^{-1}$ chloramphenicol and 100 μg $ml^{-1}$ ampicillin in a 4-l culture flask and incubate at 37 °C on a shaker until mid-log phase.

(iii) Monitor the optical density of the culture at 600 nm every 60 min. Make sure that the culture is growing exponentially. The $OD_{600}$ of the culture should increase linearly in a graph of log $OD_{600}$ versus time.

(iv) At $OD_{600}$ of 0.5, remove the flask from the shaker and chill the culture by shaking the flask in an ice water bath for 5 min.

(v) Incubate the chilled culture at 15 °C in a shaker for 45 min.

(vi) Add IPTG to a final concentration of 1 mM to induce both MazF and the protein of interest from the pCold vector. Harvest cells from 1.5 ml of the culture by centrifugation (12,000*g*, 5 min, 4 °C) and store them at –20 °C to examine the expression level by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).

(vii) Continue the culture at 15 °C with shaking. The incubation period required for optimal protein expression should be determined in a pilot experiment. The incubation period may vary from 1 to 4 d.

(viii) Harvest cells from 1.5 ml of the culture by centrifugation (12,000*g*, 5 min, 4 °C) and store them at –20 °C to examine the expression level by SDS-PAGE. Harvest the cells from rest of culture by centrifugation (5,000*g*, 15 min, 4 °C) for purification of the protein.

(ix) Examine the expression level of the target protein and its purity by SDS-PAGE.
▲ **CRITICAL STEP** Cells from 300 μl of the culture is analyzed by SDS-PAGE followed by Coomassie blue staining. For monitoring the expression level, cells before target protein expression ['0-time', Step 14A(vi)] are used as a control. Cells transformed with the empty pCold vector and treated in parallel to the experimental expression construct is an efficient control instead of many 0-time controls, especially for checking a large number of target protein expression constructs in a small-scale pilot experiment. For monitoring the progress of purification, a fraction of culture from each step may be used as references during SDS-PAGE. Remember to include molecular weight markers.

**(B) Incorporation of selenomethionine or other amino acid analogs**

(i) Pick a single colony (containing pMazF and the expression plasmid), using a toothpick, from freshly plated transformed cells in Step 13 and grow overnight in 50 ml M9-CAA medium containing 25 μg $ml^{-1}$ chloramphenicol and 100 μg $ml^{-1}$ ampicillin at 37 °C on a shaker (at approximately 150 r.p.m.).

(ii) Centrifuge (5,000*g*, 15 min, 25 °C) the overnight culture to remove the culture medium.

(iii) Resuspend the cell pellet in 10 ml M9 medium.
▲ **CRITICAL STEP** CAA should not be added, as incorporation efficiency of amino acid analogs is very poor in the presence of CAA.

(iv) Add the cell suspension to 1-l M9 medium containing 25 μg $ml^{-1}$ chloramphenicol and 100 μg $ml^{-1}$ ampicillin in a 4-l culture flask and incubate at 37 °C on a shaker.

(v) Monitor the optical density of the culture at 600 nm every 60 min. Make sure that the culture is growing exponentially. The $OD_{600}$ of the culture should increase linearly in a graph of log $OD_{600}$ versus time.

(vi) At $OD_{600}$ of 0.5, remove the flask from the shaker and chill the culture by shaking the flask in an ice water bath for 5 min.

(vii) Incubate the chilled culture at 15 °C in a shaker for 45 min.

(viii) Centrifuge (5,000*g*, 15 min, 15 °C) the culture to collect the cells.

(ix) Resuspend the cell pellet in 25-ml M9 medium containing Lys (100 μg $ml^{-1}$), Phe (100 μg $ml^{-1}$), Thr (100 μg $ml^{-1}$), Ile (50 μg $ml^{-1}$), Leu (50 μg $ml^{-1}$), Val (50 μg $ml^{-1}$), 25 μg $ml^{-1}$ chloramphenicol and 100 μg $ml^{-1}$ ampicillin (this will condense the cell culture 40-fold).
▲ **CRITICAL STEP** Other amino acid analogs (such as fluorophenylalanine) may be directly added at this step, if there is no appropriate way to block the biosynthesis of the target amino acid. A strain that is an auxotroph for a specific amino acid (for example, a *phe*$^-$ strain) may be used. In this case, cells should be washed with M9 medium after Step 14B(viii) to remove the amino acid added in the medium and recentrifuged before proceeding.

(x) Transfer the culture into a 250-ml culture flask and incubate at 15 °C with shaking for 30 min to inhibit endogenous Met biosynthesis[22].

(xi) Add 250 µl 6 mg ml$^{-1}$ seleno-L-methionine (final concentration 60 µg ml$^{-1}$) and 25 µl 1 M IPTG (final concentration 1 mM) to the culture. Harvest cells from 37.5 µl of the culture by centrifugation (12,000*g*, 5 min, 4 °C) and store them at –20 °C to examine the expression level by SDS-PAGE.

(xii) Incubate the culture at 15 °C with shaking for 12 more hours.

(xiii) Harvest cells from 37.5 µl of the culture by centrifugation (12,000*g*, 5 min, 4 °C) and store them at –20 °C to examine the expression level by SDS-PAGE. Harvest the cells from rest of culture by centrifugation (5,000*g*, 15 min, 4 °C) for purification of the protein.

(xiv) Examine the expression level of the target protein and its purity by SDS-PAGE.

▲ **CRITICAL STEP** Cells from 300 µl of noncondensed culture or 7.5 µl 40-fold condensed culture is analyzed by SDS-PAGE followed by Coomassie blue staining. For monitoring the expression level, cells prior to target protein expression ['0-time', Step 14B(xi)] are used as a control. For monitoring the progress of purification, a fraction of culture from each step may be used as references during SDS-PAGE. Remember to include molecular weight markers.

**(C) Incorporation of isotopes or isotope-labeled amino acids**

(i) Pick a single colony (containing pMazF and the expression plasmid), using a toothpick, from freshly plated transformed cells in Step 13 and grow overnight in 50-ml M9-CAA medium containing 25 µg ml$^{-1}$ chloramphenicol and 100 µg ml$^{-1}$ ampicillin at 37 °C on a shaker (at approximately 150 r.p.m.).

(ii) Centrifuge (5,000*g*, 15 min, 25 °C) the overnight culture to remove the culture medium.

(iii) Resuspend the cell pellet in 10 ml M9 medium.

▲ **CRITICAL STEP** CAA should not be added, as incorporation efficiency of isotopes or isotope-labeled amino acid is very poor in the presence of CAA.

(iv) Add the cell suspension to 1-l M9 medium containing 25 µg ml$^{-1}$ chloramphenicol and 100 µg ml$^{-1}$ ampicillin in a 4-l culture flask and incubate at 37 °C on a shaker.

(v) Monitor the optical density of the culture at 600 nm every 60 min. Make sure that the culture is growing exponentially. The $OD_{600}$ of the culture should increase linearly in a graph of log $OD_{600}$ versus time.

(vi) At $OD_{600}$ of 0.5, remove the flask from the shaker and chill the culture by shaking the flask in an ice water bath for 5 min.

(vii) Incubate the chilled culture at 15 °C in a shaker for 45 min.

(viii) Add IPTG to a final concentration of 1 mM to induce expression of both MazF and the protein of interest. Harvest cells from 1.5 ml of the culture by centrifugation (12,000*g*, 5 min, 4 °C) and store them at –20 °C to examine the expression level by SDS-PAGE.

(ix) Continue the culture at 15 °C with shaking for 3 more hours.

▲ **CRITICAL STEP** This 3-h preincubation before isotope labeling is important to eliminate isotope incorporation into background cellular proteins.

(x) Centrifuge (5,000*g*, 15 min, 15 °C) the culture to collect the cells and wash the cells once with 100 mM phosphate buffer (pH 7.5).

(xi) Resuspend the cell pellet in 25 ml of M9 medium containing 25 µg ml$^{-1}$ chloramphenicol, 100 µg ml$^{-1}$ ampicillin, 1 mM IPTG and appropriate isotopes (this will condense the cell culture 40-fold) and transfer the cell suspension into a 250-ml culture flask. Harvest cells from 37.5 µl of the culture by centrifugation (12,000*g*, 5 min, 4 °C) and store them at –20 °C to examine the expression level by SDS-PAGE.

▲ **CRITICAL STEP** For $^{15}N$ labeling experiments, $NH_4Cl$ in the M9 medium is replaced with $^{15}NH_4Cl$. For $^{15}N$ and $^{13}C$ double labeling experiments, $NH_4Cl$ and glucose in the M9 medium are replaced with $^{15}NH_4Cl$ and $^{13}C$-glucose.

(xii) Incubate the condensed culture at 15 °C with shaking for 12 more hours. Note that the incubation period may be considerably shorter or longer depending on the purpose of the experiment.

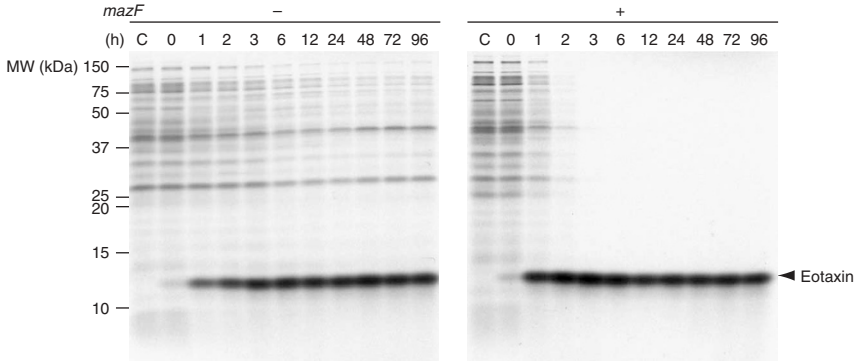


**Figure 3** | Examination of the signal-to-noise ratio for protein production in the single protein production (SPP) system. Human eotaxin, a 74-residue chemokine, is produced in the SPP system. The experiment was carried out as described in the text. Cells were pulse-labeled with $^{35}S$-Met for 15 min before (lane C) or after MazF induction at the time points indicated. Left panel; *Escherichia coli* BL21(DE3) cells transformed only with pColdI(SP-2)eotaxin were used. Right panel; *E. coli* BL21(DE3) cells transformed with pACYC*mazF* and pColdI(SP-2)eotaxin[5] were used. Molecular weight markers are shown on the left.

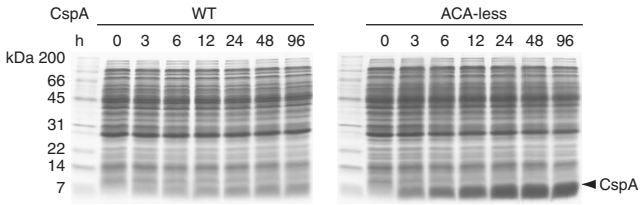


**Figure 4 |** Efficiency of protein production in the single protein production (SPP) system. Wild-type (WT) (left panel) and ACA-less CspA (right panel) are expressed in the SPP system using pColdIV (SP-2)*cspA* (WT or ACA-less), together with pACYC*mazF*. Protein induction was carried out as described in the text, and the experiments were followed by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining. Molecular weight markers are shown on the left. The position of CspA is shown by an arrow on the right.

(xiii) Harvest cells from 37.5 µl of the culture by centrifugation (12,000*g*, 5 min, 4 °C) and store them at −20 °C to examine the expression level by SDS-PAGE. Harvest the cells from rest of culture by centrifugation (5,000*g*, 15 min, 4 °C) for purification of the protein.

▲ **CRITICAL STEP** For NMR studies, cell lysates may be used without purification.

(xiv) Examine the expression level of the target protein and its purity by SDS-PAGE.

▲ **CRITICAL STEP** Cells from 300 µl noncondensed culture or 7.5 µl of 40-fold condensed culture are analyzed by SDS-PAGE for Coomassie blue staining. For monitoring the expression level, cells before the expression of the target protein (Step viii) and 0-time point for isotope labeling (Step xi) are used as controls. Remember to include molecular weight markers.

## ● TIMING

Steps 1–3, creation of ACA-less genes: 2–15d
Steps 4–9, cloning the ACA-less gene into the pCold vector: 2–5 d
Steps 10–13, preparation of cells for expressing protein: 5–7 d
Step 14, induction of protein expression: 2 d and reaction continues for 1 week

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

**TABLE 1 |** Troubleshooting table.

| Problem | Cause | Solution |
|---|---|---|
| Background protein synthesis is not eliminated after inducing MazF expression or is detected at some time point | Contamination | Each step should be done carefully to avoid contamination |
| | | Sterilize all equipment used for culture |
| | Mutation in the *mazF* gene | Retransform pMazF and use freshly transformed cells |
| No target protein expression | Duration of expression period is not optimized | Try small-scale pilot experiment to determine appropriate time duration |
| | Expression level is low even without MazF expression | Use expression system which can express target protein without MazF expression |
| No target protein expression after condensing culture | Culture condensed too much | Try small-scale pilot experiment to determine optimal factor for condensing culture |
| Cells do not grow before inducing MazF expression | Cells are not fresh | Use freshly transformed cells |
| | Wrong medium | Prepare medium again |
| Cells grow after inducing MazF expression | Contamination | Each step should be done carefully to avoid contamination |
| | | Sterilize all equipment used for culture |
| | Mutation in the *mazF* gene | Retransform pMazF and use freshly transformed cells |
| No colonies after transformation | Competent cells are too old | Prepare new batch of competent cells |

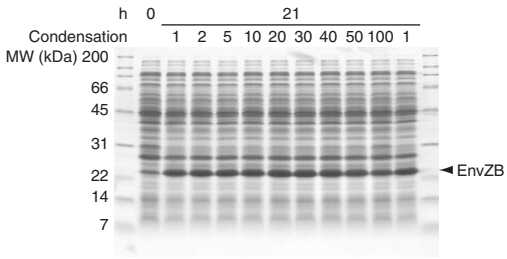


**Figure 5 |** Expression cultures can be highly condensed without sacrificing yield. ACA-less EnvZB was expressed from pColdI(SP-4) along with MazF from pACYC*mazF*. Cultures were grown to an $OD_{600}$ of 0.5, shifted to 15 °C for 45 min, concentrated to the levels shown and then EnvZB was induced with isopropyl-β-D-thiogalactopyranoside for 21 h in M9 medium. Samples were subjected to SDS–polyacrylamide gel electrophoresis followed by Coomassie blue staining. Molecular weight markers are shown on the left; the position of EnvZB is designated by an arrow to the right.

## ANTICIPATED RESULTS

### Examination of the signal-to-noise ratio

The best method to examine the signal-to-noise ratio for isotope-labeling experiments in the SPP system is to compare $^{35}$S-Met incorporation into the protein to be expressed with that into background cellular proteins. **Figure 3** shows $^{35}$S-Met incorporation into human eotaxin, a 74-residue chemokine, using the SPP system with (right panel) and without (left panel) induced MazF. It clearly demonstrates that the background cellular protein synthesis almost completely disappears 3 h after MazF induction. In this experiment, the ACA-less eotaxin gene was chemically synthesized with the *E. coli* optimum codon usage and cloned into pColdI(SP-2), a prototype of pColdI(SP-4) that contains one ACA sequence in the 3′-untranslated region.

As can be seen in **Figure 3**, upon MazF induction, a very high signal-to-noise ratio is obtained for eotaxin, which is maintained at least for 96 h. This exclusive production of a protein from an ACA-less gene has been observed for many other proteins from human, yeast to bacteria[5]. It has also been shown that the addition of ACA-sequences to these ACA-less genes dramatically reduces the $^{35}$S-Met incorporation into the respective proteins[5].

### High yield of protein production

As expected from the efficient $^{35}$S-Met incorporation into a protein of interest in the SPP system (**Fig. 3**), the amount of protein produced in the SPP system may reach as high as 20–30% of total cellular proteins[5]. **Figure 4** shows Coomassie blue staining of a gel analyzing total cellular protein expression during a 96-h incubation of cells using the SPP system. An ACA-less *cspA* gene is expressed from pColdIV(SP-2) in this system (right panel). This gene encodes the major cold-shock protein and contains three ACA sequences in the wild type. Therefore, the wild-type *cspA* gene is hardly expressed (left panel). On the other hand, the ACA-less *cspA* gene is consistently expressed and CspA protein (indicated by an arrow) accumulates over the 96-h incubation period. The final yield was estimated at approximately 25% of total cellular proteins. Note that, in these experiments, the same amount of culture was used for the SDS-PAGE analysis. Therefore, the fact that the density of all the cellular protein bands was almost unchanged during the 96-h incubation (for both right and left panels) clearly confirms the effectiveness of the SPP system, which completely blocks cellular protein synthesis.

The cSPP system achieves not only a high yield of protein production, but also effective reduction in the cost of chemicals used for isotope labeling of proteins. **Figure 5** shows the effect of culture condensation (X1–X100) on the protein yield. Cell lysate from 300 µl of the noncondensed culture was applied to SDS-PAGE in the first lane. The equivalent amount of cells from the condensed cultures was applied to each well for examination of protein yields for condensed cultures. For EnvZB production, condensing the culture up to 40 times does not affect the protein yield, giving a 97.5% reduction in the cost of protein production. Approximately, 1.5 mg EnvZB protein was obtained from 1 ml of the 40-times-condensed culture.



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