# Chapter 13

# Site-Specific Cross-Linking of In Vitro Synthesized *E. coli* Preproteins for Investigating Transmembrane Translocation Pathways

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## Abstract

A method is described for the preparation and usage of an *E. coli* cell-free translation system primed to incorporate the commercially available photoreactive analogue of phenyalanine, *p*Bpa, into newly synthesized proteins. Incorporation is achieved by means of an amber suppressor tRNA specifically charged with *p*Bpa. The method is exemplified for the site-specific photocross-linking of the signal sequence of a Tat (twin-arginine translocation) precursor protein to the Tat translocase in the cytoplasmic membrane of *E. coli*.

**Key words:** Site-specific cross-linking, photoprobes, *p*-benzoyl-phenylalanine, amber suppressor, twin-arginine translocation, Tat, protein export, in vitro transcription-translation system, inner membrane vesicles, *Escherichia coli*.

## 1. Introduction

During the past 20 years, site-specific photocross-linking has repeatedly been used to probe the molecular environment of secretory and membrane proteins during their synthesis, transport, and membrane integration. Site-specific photocross-linking of proteins involves the targeted introduction of photoreactive derivatives of amino acids into proteins. This is in contrast to methods of chemical cross-linking, in which for example amino group-specific bifunctional compounds can cross-link essentially any free amino group of a protein to its nearest neighbours.

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The common principle of various strategies of photocross-linking developed over the years was the incorporation of photoreactive derivatives of lysine or phenylalanine into the protein of interest during its synthesis in cell-free translation systems. Irradiation with UV light would then result in the formation of a covalent bond between the newly synthesized secretory or membrane protein and any contacting component, be it of cytosolic or membranous origin. Initial protocols made use of chemically modifying the ε-amino group of lysine with azido-(nitro)-benzoyl or trifluoromethyldiazirino-benzoyl moieties. Modifications were performed after the lysine had been charged onto isolated tRNA. In this approach, positioning of the photoprobe was restricted to the places of naturally occurring lysine codons in the mRNA that coded for the protein of interest. A more versatile strategy allowing a wider selection of photoprobe positions was the use of amber stop codon-suppressing tRNAs that were chemically charged with trifluoromethyldiazirine-phenylalanine (Tmd-Phe). In this way, the photoprobe could be placed at any position in the polypeptide chain, whose corresponding codon had been replaced by the amber stop codon TAG.

When applied to the study of eukaryotic secretory and membrane proteins, these photocross-linking strategies revealed the 54 kDa-subunit of the signal recognition particle (SRP), the α-subunit of the Sec61 translocon, the TRAM-protein (translocating chain-associating membrane protein) of the endoplasmic reticular membrane, as well as phospholipids as interacting partners of ribosome-bound, nascent secretory and membrane proteins (1–6). These findings could be recapitulated for bacterial secretory and membrane proteins using the same photocross-linking protocols, the only exception being that membrane proteins integrating into the bacterial cytoplasmic membrane contact YidC instead of the eukaryote-specific TRAM-protein (7–10).

Site-specific photocross-linking was recently also applied to investigate what is called the twin-arginine translocation (Tat)-pathway of bacteria. The Tat-pathway (11–14) is dedicated to the export of secretory proteins harbouring an almost invariant twin-arginine sequence motif in their N-terminal signal peptides. Moreover, it has the remarkable ability to export proteins in a fully folded conformation. In many bacteria, the Tat-specific export is achieved by three functionally individual membrane proteins, termed TatA, TatB, and TatC. TatC and TatB form a complex that is involved in recognition of the Tat-signal sequences and their insertion into the membrane. TatA is believed to mediate the actual translocation event, but it is virtually unclear what kind of protein-conducting device the TatABC proteins provide.

To investigate this in more detail, we have performed photocross-linking to pick up interactions between a Tat-substrate protein and the individual subunits TatA, TatB, and

TatC (15, 16). Initially Tmd-Phe was used which, however, has the considerable disadvantage of not being commercially available and requiring numerous steps of chemical synthesis part of which give only little yield of product. A technically much simpler and more easily applicable approach of acylating the amber suppressor tRNA directly with a photoreactive analogue of phenylalanine became recently available. The Schultz lab constructed plasmids that encode the orthogonal pair of an amber suppressor tRNA, which specifically accepts the photoreactive derivative of Phe, *p*-benzoyl-L-phenylalanine (*p*Bpa), as well as its cognate *p*Bpa-specific amino acyl-tRNA synthetase (17). Here we describe how cell-free extracts can be prepared from *E. coli* strains harbouring these plasmids and therefore expressing the pair of *p*Bpa-specific amber suppressor tRNA/tRNA-synthetase. We further detail how these extracts can be used to incorporate externally added *p*Bpa into an amber mutant Tat-substrate protein and how this is employed for efficient photocross-linking to Tat subunits present in membrane vesicles of *E. coli*.

## 2. Materials

### *2.1. Preparation of an Amber Stop Codon-Suppressing S-135 Cell Extract from* E. coli

1. Growth medium (S-30 medium): 9.0 g/L tryptone/peptone (pancreatic digest of casein; Carl Roth, Karlsruhe, Germany), 0.8 g/L yeast extract, 5.6 g/L NaCl, 1 mL/L 1 M NaOH. Prepare 4–6 L in 1-L batches, each contained in a 5-L Erlenmeyer flask covered with aluminium foil and autoclave. Prepare an additional 100 mL of medium in a 0.5 L Erlenmeyer flask to be used as starter culture and autoclave (*see* **Note 1**).
2. 5 mg/ml Tetracycline in 70% (v/v) ethanol p.a. stored in 1-ml aliquots at −20°C (*see* **Note 2**).
3. 20% Glucose solution, autoclaved.
4. 1 M Triethanolamine acetate (TeaOAc) adjusted to pH 7.5 with acetic acid, filtered and stored at 4°C (*see* **Note 3**).
5. 1 M Magnesium acetate ($Mg(OAc)_2$), filtered and stored at 4°C.
6. 4 M Potassium acetate (KOAc) also adjusted to pH 7.5 with acetic acid, filtered and stored at 4°C.
7. 1 M Dithiothreitol (DTT) stored in 1-mL aliquots at −20°C.
8. S-30 buffer: 10 mM TeaOAc pH 7.5, 14 mM $Mg(OAc)_2$, 60 mM KOAc, 1 mM DTT, stored at 4°C.

9. Phenylmethylsulfonyl fluoride (PMSF; Roche): freshly prepare about 1 mL of a 0.1 M solution in ethanol before use (*see* **Note 4**).
10. A mix of 18 amino acids (without methionine and cysteine) in water each at a concentration of 1 mM.
11. 1 mM Methionine.
12. 1 mM Cysteine.
13. 0.25 M ATP neutralized with 1 M KOH.
14. 0.2 M Phosphoenol pyruvate tri(cyclohexylammonium) salt.
15. 2 mg/mL Pyruvate kinase solution (Roche).
16. Supplemented S-30 (for degradation of endogenous mRNA): per mL of S-30, add 60 μL 1M TeaOAc pH 7.5, 0.6 μL 1M DTT, 1.6 μL 1 M $Mg(OAc)_2$, 6 μL 1 mM 18 amino acid mix, 6 μL 1 mM methionine, 6 μL 1 mM cysteine, 2 μL 0.25 M ATP (neutralized), 27 μL 0.2 M phosphoenol pyruvate, and 2.4 μL 2 mg/mL pyruvate kinase.
17. Dialysis tubing with a width of 25 mm and a molecular weight cut off of 14,000 Da (Visking; Carl Roth) (*see* **Note 5**). Two dialysis tubing clips.
18. For preparation of dialysis tubing: 2% $NaHCO_3$, 1 mM ethylenediamine tetraacetic acid (EDTA).

### *2.2. Preparation of Inverted Inner Membrane Vesicles (INV)*

1. Growth Medium (INV medium): 10 g/L each of yeast extract and tryptone/peptone (pancreatic digest of casein; Carl Roth), 28.9 g/L $K_2HPO_4$ anhydrous, 5.6 g/L $KH_2PO_4$ anhydrous, 10 g/L glucose. Prepare 4 × 5-L Erlenmeyer flasks, each containing 10 g yeast extract and 10 g tryptone/peptone dissolved in 753 mL $H_2O$, autoclave. In addition, prepare one 0.5-L Erlenmeyer flask containing 1 g yeast extract and 1 g tryptone/peptone dissolved in 75.3 mL $H_2O$, autoclave.
2. 1 M $K_2HPO_4$, autoclave.
3. 1 M $KH_2PO_4$, autoclave.
4. 25% Glucose, autoclave.
5. Starter culture medium (100 mL): to 75.3 mL yeast extract and tryptone/peptone (*see* **Section 2.2**, item 1) add 4.1 mL 1 M $KH_2PO_4$, 16.6 mL 1 M $K_2HPO_4$, and 4 mL 25% glucose.
6. Complete INV medium: to 753 mL yeast extract and tryptone/peptone (*see* **Section 2.2**, item 1) add 41 mL 1 M $KH_2PO_4$, 166 mL 1 M $K_2HPO_4$, and 40 mL 25% glucose.

7. 1 M TeaOAc adjusted to pH 7.5 with acetic acid, filtered and stored at 4°C.
8. 0.2 M EDTA-KOH, pH 7.0, filtered and stored at 4°C.
9. 2.5 M Sucrose ultrapure (MP Biomedicals, Solon, OH), heat slightly for better dissolution, store at room temperature.
10. 1 M DTT stored in 1-mL aliquots at −20°C.
11. 0.1 M PMSF freshly prepared in ethanol.
12. 1 M Isopropyl-β-D-thiogalactopyranoside (IPTG).
13. Buffer A: 50 mM TeaOAc, pH 7.5, 250 mM sucrose, 1 mM EDTA-KOH, pH 7.0, and 1 mM DTT. Prepare fresh.
14. Buffer B: 0.5 M TeaOAc, pH 7.5, 10 mM EDTA-KOH, pH 7, and 10 mM DTT. Prepare fresh.
15. Sucrose solutions for sucrose gradient centrifugation, freshly prepared. 0.77 M sucrose: 10 mL buffer B, 30.8 mL 2.5 M sucrose, $H_2O$ to 99.5 mL, 0.5 mL 0.1 M PMSF added last; 1.44 M sucrose: 10 mL buffer B, 57.6 mL 2.5 M sucrose, $H_2O$ to 99.5 mL, 0.5 mL 0.1 M PMSF added last; 2.02 M sucrose: 10 mL buffer B, 80.8mL 2.5 M sucrose, $H_2O$ to 99.5 mL, 0.5 mL 0.1 M PMSF added last.
16. INV buffer: 50 mM TeaOAc, pH 7.5, 250 mM sucrose, and 1 mM DTT. Cool on ice.

### *2.3. Site-Specific Cross-Linking of In Vitro Synthesized* E. coli *Precursor Proteins Using a pBpa-Specific Amber Suppressor tRNA and Its Cognate Amino Acyl-tRNA Synthetase*

#### *2.3.1. In Vitro Transcription-Translation Reaction and Site-Specific Cross-Linking*

1. Template DNA: Plasmid DNA prepared by Qiagen plasmid maxi kit is suitable for in vitro synthesis (*see* **Note 6**). Prepare DNA in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at about 1 μg/μL and store at 4°C. For site specific incorporation of the photo-reactive cross-linker *p*-benzoyl-*L*-phenylalanine (*p*Bpa), introduce at selected positions TAG stop codons into the DNA sequence encoding the protein of interest (*see* **Note 7**). This can be done for instance by using the PCR-based QuikChange Site-Directed Mutagenesis Kit system (Stratagene, Cedar Creek, TX, USA) following the manufacturer's instruction.
2. 1 M TeaOAc adjusted to pH 7.5 with acetic acid, filtered and stored at 4°C.
3. 4 M KOAc also adjusted to pH 7.5 with acetic acid, filtered and stored at 4°C.
4. 1 M $Mg(OAc)_2$, filtered and stored at 4°C.
5. 25 mM $Mg(OAc)_2$, filtered and stored at 4°C.
6. 0.1 M Spermidine trihydrochloride (Sigma, St. Louis, MO), dissolved in water and stored in single-use aliquots at −20°C (*see* **Note 8**).

7. 40% (w/v) Polyethylene glycol 6000–8000, dissolved in water and stored in 1-mL aliquots at −20°C.
8. 1 mM (each) of 18 amino acids (without methionine and cysteine), dissolved in water and stored in 1-mL aliquots at −20°C.
9. 0.2 M D TT, dissolved in water and stored in 10-μL aliquots at −20°C (*see* **Note 8**).
10. 0.2 M Phosphoenol pyruvate, dissolved in water and stored in 50-μL aliquots at −20°C (*see* **Note 8**).
11. 0.5 M Creatine phosphate, dissolved in water and stored in 10-μL aliquots at −20°C.
12. 10 mg/mL Creatine phosphokinase, dissolved in water and stored in 10-μL aliquots at −20°C.
13. Neutralized nucleotide (NTP) stock (50 mM ATP and 10 mM each of GTP, CTP, UTP): prepare by mixing equal volumes of 250 mM ATP, 50 mM each of GTP, CTP, UTP, and 1 M KOH. Make all solutions in water and store the NTP stock in 10-μL aliquots at −20°C (*see* **Note 9**).
14. EasyTag$^{TM}$ Express [$^{35}$S]-Protein Labelling Mix, 407 MBq (11 mCi)/mL (Perkin Elmer, USA). This mixture contains 73% [$^{35}$S]-methionine and 22% [$^{35}$S]-cysteine; store in 50-μL aliquots at −80°C. (Radioactive material is hazardous. Avoid ingestion or contact with skin or clothing. Always wear gloves when handling. Monitor hands, equipment, and bench frequently.)
15. T7 RNA Polymerase. Commercially available preparations (e.g. from Promega, Madison, WI, USA) are fine; large quantities are also reasonably easy to prepare from overproducing *E. coli* strains (18).
16. 2 mM *p*Bpa (H-p-Bz-Phe-OH, Bachem AG, Switzerland): immediately before use, prepare a fresh solution of 1 M *p*Bpa in 1 *N* NaOH and dilute to 2 mM with water. Keep on ice before use and protect from light (*see* **Note 10**).
17. 11 mM Puromycin neutralized with 1 M KOH, stored in 20-μL aliquots at −20°C.
18. 10% Trichloroacetic acid: prepare a 100% solution and use for further dilutions.
19. UV-lamp, $\lambda = 365$ nm , 6 W (e.g. VL-6.L, Vilbert Lourmat Deutschland GmbH, Germany).

*2.3.2. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

1. Separating gel buffer: 2 M Tris-HCl, pH 8.8. Filter and store at 4°C.
2. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8. Filter and store at 4°C.

3. 25% (w/v) SDS. Store at room temperature.
4. 30% Acrylamide/0.8% bisacrylamide solution (Rotiphorese Gel 30, Carl Roth). Acrylamide is a neurotoxin; always wear gloves when handling acrylamide solutions and gels.
5. *N,N,N′,N′*-Tetramethylethylene diamine (TEMED).
6. Ammonium peroxodisulfate. Prepare 10% solution in water and store at 4°C. It is stable for several days.
7. Running buffer (5x): dissolve 150 g Tris base and 720 g glycine in 5 L water and store at room temperature.
8. Solution 1: 2 mL 1 M Tris base, 1 mL 0.2 M EDTA, pH 8.0, 7 mL water.
9. Solution 2: 4 mL 25% SDS, 1 mL 1 M Tris base, 3.5 mL 100% glycerol, 3.5 mL 0.1% bromophenol blue.
10. Solution 3: 1 M DTT.
11. Prepare PAGE-loading buffer: mix five parts of solution 1, four parts of solution 2, and one part of solution 3. Always prepare fresh.
12. Prestained molecular weight marker: Precision Plus Protein Standards (Bio-Rad, Hercules, CA).
13. Fixing solution: 35% ethanol, 10% acetic acid.

## 3. Methods

### *3.1. Preparation of an S-135 Cell Extract from* E. coli *for In Vitro Synthesis and Site-Specific Cross-Linking*

1. Pre-cool the French press cell by placing it at 4°C.
2. Supplement 100 mL of S-30 medium with 0.4 mL autoclaved 20% glucose solution and 0.5 mL of 5 mg/mL tetracycline. Inoculate from plates or glycerol stocks with an *E. coli* strain carrying plasmid pDULE-pBpa (*see* **Note 11**). Grow cells overnight at 37°C with sufficient aeration in a rotary shaker (cover flask with aluminium foil). Use this culture to inoculate at a 1:100 ratio 4–6 L growth medium supplemented with 4 mL/L of 20% glucose and 0.5 mL/L of 5 mg/mL tetracycline (**see Note 12**) and grow the cells in a rotary shaker to late log-phase (optical density at 600 nm = 1.0–1.2U/mL).
3. Prepare 4 L of S-30 buffer and store at 4°C.
4. Chill the cell cultures quickly by placing the flasks in an ice water bath and harvest the cells at 4°C in a cooled SCL3000 rotor (Sorvall) for 10 min at 8650 g (7000 rpm). All subsequent steps should be done at 4°C or on ice. Resuspend the cell pellets in S-30 buffer (*see* **Note 13**).

Combine the cell suspensions in one or two tared centrifuge bottles and centrifuge again. Determine the wet weight of the cell pellet (approx 2 g/L medium).

5. Resuspend the cell pellet in 1 volume (1 mL/g wet cell mass) of S-30 buffer containing 0.5 mM PMSF (add PMSF from a fresh 0.1 M stock in ethanol).
6. For breakage of the cells pass the cell suspension two to three times through a French pressure 40 k cell (Spectronic Unicam, Cambridge, UK) at 8000 psi. This corresponds to a gage pressure setting of 500 when using the 1-in. piston cell at the "high ratio" selection (*see* **Note 14**).
7. After cell breakage centrifuge the suspension in a pre-cooled SS34 rotor (Sorvall) for 30 min at 30,000 g (15,500 rpm) at 4°C. Remove supernatant (S-30) carefully (*see* **Note 15**).
8. To allow degradation of endogenous mRNA in the S-30, perform a readout of polysomal mRNA. To this end prepare supplemented S-30 according to **Section 2.1**, item 16. Incubate at 37°C for 1 h. Afterwards chill the S-30 on ice.
9. Dialyze the S-30 three times against 1 L of cold S-30 buffer for 1 h each at 4°C (*see* **Note 16**).
10. Prepare S-135 from the S-30 (*see* **Note 17**) by pipetting 1-mL aliquots of S-30 into tubes of a Beckman TLA 100.2 rotor and spin at 287,600 g (90,000 rpm) for 13 min at 4°C. Remove 750 μL (*see* **Note 18**) of each supernatant, combine (= S-135), and quick-freeze in aliquots of 50- to 100-μL in liquid nitrogen (*see* **Note 19**). Store the S-135 at −80°C.

### *3.2. Preparation of INV from a TatABC-Overproducing* E. coli *Strain*

1. Pre-cool the French press cell by placing it at 4°C.
2. Inoculate 100 mL of starter culture medium from plates or glycerol stocks with an *E. coli* strain harbouring the *tatABC* genes cloned under an inducible promoter (*see* **Note 20**). Grow cells overnight at 37°C with sufficient aeration in a rotary shaker (cover flask with aluminium foil).
3. Inoculate 4 flasks containing 1 L complete INV medium with 20 mL of starter culture each. Grow cells at 37°C. Expression of the TatABC proteins is induced at an optical density of 0.5 by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and growth is continued for 3–4 h or until an optical density at 600 nm of 1.5–1.8 is reached (*see* **Note 20**).
4. Prepare 50 mL buffer A and cool on ice.

5. Chill the cell cultures quickly by placing the flasks in an ice water bath and harvest the cells at 4°C in a cooled SCL3000 rotor (Sorvall) for 10 min at 8650 g (7000 rpm). All subsequent steps should be done at 4°C or on ice. Resuspend the cell pellets in buffer A (*see* **Note 13**). Combine the cell suspensions to one or two tared centrifuge bottles and centrifuge again. Determine the wet weight of the cell pellet (*see* **Note 21**).
6. Resuspend the cell pellet in 1 volume (1 mL/g wet cell mass) of buffer A containing 0.5 mM PMSF (add PMSF from a fresh 0.1 M stock in ethanol).
7. For breakage of the cells, pass the cell suspension two to three times through a French pressure 40 k cell at 8000 psi. This corresponds to a gage pressure setting of 500 when using the 1-in. piston cell at the "high ratio" selection (*see* **Note 14**).
8. To remove cell debris, the extract is centrifuged for 5 min in a pre-cooled SS34 rotor (Sorvall) at 1954 g (5000 rpm) at 4°C.
9. The supernatant is collected and centrifuged again for 2 h at approx 150,000 g (40,000 rpm) in a Beckman 50.2Ti Rotor at 4°C to obtain a crude membrane pellet encompassing outer and inner membranes and ribosomes (*see* **Note 22**). The sticky pellets are carefully resuspended in buffer A by using a loosely fitting glass homogenizer (Fischer Scientific) to give a total volume of 8 mL. Crude membranes can be stored at −80°C after quick-freezing in liquid nitrogen.
10. Because of the different densities, the inner membrane vesicles can be separated from outer membranes and unbound ribosomes by sucrose gradient centrifugation. Prepare six sucrose gradients each one consisting of 12 mL 0.77 M, 12 mL 1.44 M and 10 mL 2.02 M sucrose solution in polyallomer centrifuge tubes (38.5 mL, 25 × 89 mm, Herolab centrifuge labware). Start with the 0.77 M sucrose cushion and always underlay the denser solutions by using a smoothly running syringe equipped with a horizontally cut, wide-bore needle. Equilibrate the gradients at 4°C for about 1 h. Finally, load 2–2.5 mL of the crude membranes on top of each gradient and spin at 4°C for at least 16 h at approx 81,500 g (25,000 rpm) in a swing-out rotor, type Sorvall AH 629/36.
11. After centrifugation the inner membrane fraction should be visible as a yellow layer at the interface between the 0.77 M and 1.44 M sucrose steps. Recover the membranes with a syringe by introducing the needle from the top of the

gradient or by carefully poking a hole into the tube wall at the height of the inner membrane layer. This is safely done (mind your fingers!) by use of a disposable hypodermic needle mounted on a syringe which is slowly turned clockwise and counter-clockwise between thumb and middle finger and thereby drilled across the tube wall. Push the vesicle suspension immediately into a tube placed on ice.

12. For subsequent collection, dilute the inner membrane vesicles with ice-cold 50 mM TeaOAc, pH 7.5 about fourfold (the fraction withdrawn from the gradients presumably stems to equal parts from both the 0.77 M and 1.44 M sucrose layers resulting in a calculated sucrose concentration of about 1.1 M).
13. Pellet the inner membranes by centrifugation for 2 h at approx 150,000 g (40,000 rpm) in a Beckman 50.2Ti Rotor at 4°C and carefully resuspend in INV buffer by using a loosely fitting glass homogenizer. The final desired volume of INV derived from 4 L of bacterial culture is about 1 mL. This will correspond to an absorption at 280 nm of about 30 U/mL (*see* **Note 23**).
14. Freeze the gradient-purified INV in small aliquots of about 15 μL in liquid nitrogen and store at −80°C (*see* **Note 24**).

### *3.3. Site-Specific Cross-Linking of In Vitro Synthesized* E. coli *Precursor Proteins Using a pBpa-Specific Amber Suppressor tRNA and Its Cognate Amino Acyl-tRNA Synthetase*

#### *3.3.1. In Vitro Transcription-Translation Reaction and Incorporation of pBpa via Amber Stop Codon Suppression*

1. Plan the experiment according to the table in **Fig. 13.1**: synthesize a variant of the Tat-specific precursor protein pTorA-PhoA containing *p*Bpa at position Phe15 (pTorA-PhoA-F15) by use of an amber stop codon-suppressing S-135 cell extract. Synthesis is performed either in the absence of *p*Bpa (samples 1, 3, 5, and 7) or in the presence of *p*Bpa (samples 2, 4, 6, and 8), and at $Mg^{2+}$-concentrations of either 6 mM (samples 1 and 2), 7 mM (samples 3 and 4), 8 mM (samples 5 and 6), or 9 mM (samples 7, and 8). You will need 8 × 25 μL reactions. In order to provide enough material, plan for one additional reaction, i.e., a total of 9 × 25 μL reactions.

   Next, calculate the reaction mixture as exemplified in **Table 13.1**. The reaction mixture consists of all ingredients that are common to the eight individual reactions indicated in **Fig. 13.1**. The final volume of each reaction is 25 μL. Of those, 3.5 μL are used up by the individual additives listed in **Fig. 13.1**: $H_2O$, $Mg(OAc)_2$, and *p*Bpa. In the experiment outlined here 9 × 21.5 μL = 193.5 μL reaction mixture will be prepared and subsequently distributed in 21.5 μL aliquots onto the eight reaction tubes indicated in **Fig. 13.1**.
2. Prepare 500 μL Compensating Buffer (CB) for the transcription-translation reaction on ice (*see* **Note 25**).

| Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| $H_2O$ | 3.5µL | 3µL | 2.5µL | 2µL | 1.5µL | 1µL | 0.5µL | - |
| $Mg(OAc)_2$ [25m*M*] | - | - | 1µL | 1µL | 2µL | 2µL | 3µL | 3µL |
| *p*Bpa [2m*M*] | - | 0.5µL | - | 0.5µL | - | 0.5µL | - | 0.5µL |
| Reaction mixture (see Table 1) | 21.5µL each | | | | | | | |
| Sum | 25µL each | | | | | | | |

↓

30min at 37°C

↓

20µL sample + 20µL 10% TCA each

↓

30min at ice

Fig. 13.1. In vitro incorporation of *p*Bpa into pTorA-PhoA-F15 via amber stop codon suppression. Pipetting scheme. The preparation of the reaction mix is detailed in **Table 13.1**. $Mg^{2+}$-solution and *p*Bpa solution are described in **Section 2.3.1**, items 5 and 16, respectively. TCA, trichloroacetic acid.

Efficient in vitro transcription of template DNA and translation of transcripts into protein needs defined reaction conditions. The following final concentration of ions have proven optimal for the wild type pTorA-PhoA DNA: 40 mM TeaOAc pH 7.5, 140 mM KOAc, 11 mM $Mg(OAc)_2$ (*see* **Note 26**). The optimal $Mg^{2+}$-concentration for suppression of the pTorA-PhoA-F15 amber stop codon mutant is determined in the experiment described here by titrating the $Mg^{2+}$ concentration in mM-increments. The CB therefore is prepared with 6 mM $Mg^{2+}$ as the lowest concentration intended (**Table 13.2**). In calculating the CB, the ionic contributions of the S-135 extract and plasmid DNA are taken into consideration. **Table 13.2** explains the calculation of CB for a 25 µL single reaction containing 3 µL of S-135 extract and 1 µL of plasmid DNA.

3. Thaw all required components. This is best done by placing small aliquots simply on ice and larger ones in a water bath at room temperature. Freshly prepare *p*Bpa solution (*see* **Section 2.3.1**, item 16) and protect from light. Set up a series of labelled 1.5-mL reaction tubes on ice and add $H_2O$, 25 mM $Mg(OAc)_2$, and *p*BPA as specified in **Fig. 13.1**.
4. Prepare the reaction mixture on ice according to **Table 13.1** strictly following the indicated order. Vortex before adding the first biological (creatine phosphokinase) and after the last addition, each time briefly spinning to collect all liquid at the bottom of the tube again.

**Table 13.1**
**Calculation of the reaction mixture (Section 3.3.1)**

| | Concentration of stock solution | Final concentration | µL/25 µL | µL × 9 |
|---|---|---|---|---|
| Compensating Buffer[a] | 5x | 1x | 5 | 45 |
| $H_2O$ | | up to 25 µL | 5.4 | 48.6 |
| Polyethylene glycol | 40% (w/v) | 3.2% (w/v) | 2 | 18 |
| 18 amino acids | 1 mM each | 0.04 mM each | 1 | 9 |
| DTT | 200 mM | 2 mM | 0.25 | 2.25 |
| NTP mixture:<br>(ATP<br>GTP, UTP, CTP) | <br>50 mM<br>10 mM each | <br>2.5 mM<br>0.5 mM each | 1.25 | 11.25 |
| Phosphoenol pyruvate | 200 mM | 12 mM | 1.5 | 13.5 |
| Creatine phosphate | 500 mM | 8 mM | 0.4 | 3.6 |
| Creatine phosphokinase | 10 mg/mL | 40 µg/mL | 0.1 | 0.9 |
| DNA[b] | 1 mg/mL | 40 µg/mL | 1 | 9 |
| S-135 | | | 3 | 27 |
| T7 RNA polymerase | | | 0.1[c] | 0.9 |
| [$^{35}$S]-Met/Cys | | | 0.5 | 4.5 |
| Total (= Reaction mixture) | | | 21.5 µL | 193.5 µL |
| added separately (*see* **Fig. 13.1**): | | | | |
| *p*Bpa | 2 mM | 0.04 mM | 0.5 | |
| $Mg(OAc)_2$ | 25 mM | | up to 3 | |

[a]See **Table 13.2**.
[b]DNA used here is plasmid pET28a-TorA-PhoAF15 (28).
[c]Depends on activity; use 5–10U of a commercial enzyme.

5. Subdivide the reaction mixture onto the eight reaction tubes as indicated in **Fig. 13.1**. Vortex and briefly spin to collect all liquid at the bottom of the tubes.
6. Start the reactions by incubating all tubes at 37°C for 30 min (*see* **Note 27**).
7. Spin briefly and place tubes on ice to stop the synthesis reaction.
8. Add 20 µL of each reaction to 20 µL 10% trichloracetic acid each, mix, and let it precipitate on ice for at least 30 min (*see* **Note 28**).
9. Pellet precipitated proteins by centrifugation for 10 min in a tabletop microcentrifuge at room temperature. Carefully remove supernatant by aspiration into the radioactive waste.
10. Add 30 µL PAGE-loading buffer to each sample and shake vigorously at room temperature to dissolve the pellet com-

**Table 13.2**
**Calculation of compensating buffer (CB)**

| | Tea[a]/Tris (nmol) | $K^+$ (nmol) | $Mg^{2+}$ (nmol) | Spermidine (nmol) | $H_2O$ |
|---|---|---|---|---|---|
| 3 μL S135 (10 mM Tea, 60 mM $K^+$, 14 mM $Mg^{2+}$)[b] | 30 | 180 | 42 | | |
| 1 μL DNA (10 mM Tris)[b] | 10 | | | | |
| Total (1) | 40 | 180 | 42 | | |
| Desired final concentration: 40 mM Tea, 140 mM $K^+$, 6 mM $Mg^{2+}$, 0.8 mM spermidine →nmol desired in 25-μL reaction (2) | 1000 | 3500 | 150 | 20 | |
| Difference (2)–(1) →nmol required for 25-μL reaction to be added via CB (3) | 960 | 3320 | 108 | 20 | |
| Required nmol (3) are added in 5 μL CB → required nmol/μL CB (4) (= mM concentration of CB) | 192 | 664 | 21.6 | 4 | |
| To prepare 1 mL of such CB from 1 M Tea, 4 M $K^+$, 1 M $Mg^{2+}$, 0.1 M spermidine stocks add | μL 192 | μL 166 | μL 21.6 | μL 40 | μL 580.4 |
| To prepare 500 μL of such CB Add | 96 | 83 | 10.8 | 20 | 290.2 |

[a]Tea, triethanolamine
[b]Components that contribute relevantly to the ionic composition of the reaction mixture

pletely. The colour of the loading buffer should remain dark blue. If it changes to yellow, add a few microliter of 1 M Tris base to neutralize residual trichloroacetic acid. Heat samples at 95°C for 5 min and analyse by SDS-PAGE and autoradiography.

*3.3.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Autoradiography*

1. Electrophoresis is carried out in large custom-made units. Dimensions of the gel are 35 cm × 25 cm × 1 cm (W × L × T). These gels are made from about 80 and 20 mL of separating and stacking gel solutions, respectively.
2. To prepare 100 mL of a 12% separating gel, add 40 mL acrylamide/bisacrylamide solution, 10 mL separating gel buffer, 0.4 mL 25% SDS to a measuring cylinder and adjust volume to 100mL with water. Add 0.04 mL TEMED and 0.6 mL ammonium peroxodisulfate to start polymerization, pour the solution into gel cassettes mounted in an upright position, and overlay with isobutanol.

3. After polymerization remove isobutanol, rinse with water and prepare 30 mL of stacking-gel solution by adding 5 mL acrylamide/bisacrylamide solution, 3.6 mL stacking-gel buffer, 0.12 mL 25% SDS to a measuring cylinder and adjusting the volume to 30 mL with water. Add 0.012 mL TEMED and 0.2 mL ammonium peroxodisulfate to start polymerization, pour the solution into the gel cassettes, and immediately insert a comb.
4. Prepare 2 L of running buffer by dilution from the 5x stock and addition of 8 mL 25% SDS; add to upper and lower chambers of the electrophoresis apparatus.
5. Load the samples completely into the wells of the gel and include one lane for prestained molecular weight markers. Electrophoresis is usually carried out overnight at a constant current of 20 mA until the bromophenol blue dye has run to the bottom of the gel. Avoid its running off the gel in order to retain any radioactive substance of similarly low molecular mass on the gel.
6. Remove the stacking gel and incubate the separating gel in fixing solution for 20 min on a shaking platform. Discard the fixing solution and incubate the gel in water three times for 10 min each.
7. Transfer the gel onto a prewetted Whatman 3MM paper, cover with plastic wrap, and dry at 70°C for 2 h on a vacuum dryer (Bio-Rad).
8. Expose the dried gel to a phosphorimaging screen overnight and analyse the autoradiogram on a PhosphorImager (e.g. Storm, GE Healthcare) using ImageQuant$^{TM}$ software.
9. Print an image of the autoradiogram at magnification 1 and transfer the positions of the prestained molecular weight markers to the printout. The autoradiogram of the experiment described under **Section 3.3.1** is shown in **Fig. 13.2**.

*3.3.3. In Vitro Cross-Linking of pBpa-Containing pTorA-PhoA with the TatC Subunit of the Tat Translocase of* E. coli *Inner Membrane Vesicles*

1. Plan the experiment according to **Fig. 13.3**: synthesize pTorA-PhoA-F15 carrying a photo-reactive derivative of phenylalanine at position Phe15 by use of an amber stop codon-suppressing S-135 cell extract. Synthesis is performed either in the absence of *p*Bpa (sample 3) or in its presence (samples 1, 2 and 4). You will need 8 × 25 μL reactions (2 × 25 μL for each sample). In order to provide enough material, plan for two additional reactions, i.e., a total of 10 × 25 μL reactions. Calculate the reaction mixture according to **Table 13.3**. The reaction mixture contains all ingredients needed for in vitro synthesis of pTorA-PhoA-F15 except for *p*Bpa. Of the final volume of each 25 μL reaction, 0.5 μL is used up by the

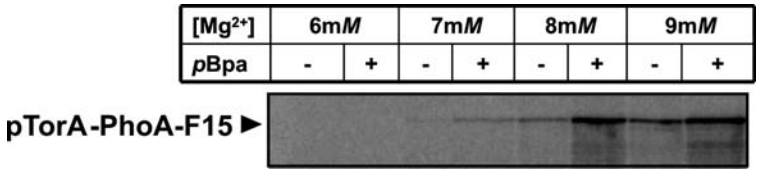


Fig. 13.2. In vitro suppression of the amber stop codon mutant pTorA-PhoAF15 by *p*-benzoyl-*L*-phenylalanine (*p*Bpa) and its dependence on the $Mg^{2+}$-concentration. Autoradiogram of the experiment outlined in **Fig. 13.1**. The precursor protein pTorA-PhoA-F15 was synthesized by a coupled in vitro transcription-translation system, which had been prepared from an *E. coli* strain transformed with plasmid pDULE-pBpa. This plasmid encodes a *p*Bpa-specific amber suppressor tRNA together with a cognate, *p*Bpa-specific amino acyl-tRNA-synthetase. The final concentration of $Mg^{2+}$ and the addition of *p*Bpa are indicated. After synthesis samples were precipitated by trichloroacetic acid. Samples were separated by SDS-PAGE and visualized by phosphor imaging. Note the suppression of the amber stop codon in the presence of *p*Bpa leading to the synthesis of full-size pTorA-PhoA-F15 at $Mg^{2+}$-concentrations >6 mM, and some inevitable read-through of the stop codon, particularly at higher $Mg^{2+}$-concentrations.

subsequent addition of *p*Bpa. In the experiment outlined here 10 × 24.5 μL = 245 μL reaction mixture will be prepared and split into a 7 × 24.5 μL = 171.5 μL aliquot (*p*$Bpa^+$-mixture) and into a 49 μL aliquot (*p*$Bpa^-$-mixture) receiving 3.5 μL *p*Bpa solution and 1 μL $H_2O$, respectively.

2. Prepare 500 μL Compensating Buffer (CB). CB is composed as illustrated in **Table 13.2** except that it is calculated with an intended $Mg^{2+}$-concentration of 8 mM according to the results shown in **Fig. 13.2**.
3. Thaw all required components. Thawing is best done by placing small aliquots simply on ice and larger ones in a water bath at room temperature. Freshly prepare *p*Bpa solution (*see* **Section 2.3.1**, item 16) and protect from light.
4. Prepare the reaction mixture on ice according to **Table 13.3** strictly following the indicated order. Vortex before adding the first biological (creatine phosphokinase) and after the last addition, each time briefly spinning to collect all liquid at the bottom of the tube again. All subsequent steps should be performed in the dark to avoid untimely activation of the photo-probe.
5. Subdivide the reaction mixture according to **Fig. 13.3** onto two new reaction tubes ($pBpa^+$-mix and $pBpa^-$-mix) and add *p*Bpa solution or $H_2O$.
6. Start the reactions by incubating both tubes at 37°C for 30 min in the dark.
7. Stop synthesis by the addition of puromycin and incubation at 37°C for 10 min in the dark.

| | *p*Bpa$^+$-mix | *p*Bpa$^-$-mix |
|---|---|---|
| Reaction mixture (see Table 3) | 171.5µL | 49µL |
| *p*Bpa | 3.5µL | - |
| $H_2O$ | - | 1µL |
| Σ Volume | 175µL | 50µL |

30min at 37°C, dark

| | |
|---|---|
| 11m*M* puromycin | 14µL / 4µL |

10min at 37°C, dark

| Sample | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Tat$^+$-INV | - | 2µL | 2µL | 2µL |
| INV-buffer | 2µL | - | - | - |
| *p*Bpa$^+$-mix | 50µL | 50µl | - | 50µL |
| *p*Bpa$^-$-mix | - | - | 50µL | - |
| Sum | 52µL each | | | |

15 min at 37°C, dark

Sample 2: 45µL sample + 45µL TCA → 30min on ice

Samples 1, 3 and 4: UV-light, 20min on ice → 45µL sample + 45µL TCA each → 30min on ice

Fig. 13.3. In vitro cross-linking of *p*Bpa-containing pTorA-PhoA to the TatC subunit of the Tat translocase of *E. coli* inner membrane vesicles. The preparation of the reaction mixture is detailed in **Table 13.3**. To uncouple synthesis of pTorA-PhoA-F15 from its binding to membrane vesicles, protein synthesis is blocked by puromycin (*see* **Section 2.3.1**, item 17). Tat$^+$-INV were prepared from strain BL21(DE3) pLysS p8737. INV-buffer is described in **Section 2.2**, item 16. TCA, trichloroacetic acid.

8. Set up a series of labelled 1.5-mL reaction tubes on ice and add Tat$^+$-INV (*see* **Note 20**) or INV buffer as indicated in the lower table of **Fig. 13.3**.
9. Subdivide both reaction mixtures onto the four reaction tubes as indicated in the lower table of **Fig. 13.3**. Vor-

**Table 13.3**
**Calculation of the reaction mixture (Section 3.3.3)**

| | Concentration of stock solution | Final concentration | µL/25µL | Reaction mix: µL x 10 |
|---|---|---|---|---|
| Compensating Buffer[a] | 5x | 1x | 5 | 50 |
| $H_2O$ | | up to 25 µL | 7.775 | 77.75 |
| Polyethylene glycol | 40% (w/v) | 3.2% (w/v) | 2 | 20 |
| 18 amino acids | 1 mM each | 0.04 mM each | 1 | 10 |
| DTT | 200 mM | 2 mM | 0.25 | 2.5 |
| NTP mixture (ATP, GTP,UTP, CTP) | 50 mM 10 mM each | 2.5 mM 0.5 mM each | 1.25 | 12.5 |
| Phosphoenol pyruvate | 200 mM | 12 mM | 1.5 | 15 |
| Creatine phosphate | 500 mM | 8 mM | 0.4 | 4 |
| Creatine phosphokinase | 10 mg/mL | 40 µg/mL | 0.1 | 1 |
| GSSG[b] | 200 mM | 5 mM | 0.625 | 6.25 |
| DNA[c] | 1 mg/mL | 40 µg/mL | 1 | 10 |
| S-135 | | | 3 | 30 |
| T7 RNA polymerase | | | 0.1[d] | 1 |
| [$^{35}$S]-Met/Cys | | | 0.5 | 5 |
| Total (= reaction mixture) | | | 24.5 µL | 245 µL |
| added separately (see **Fig.13.3**): | | | | |
| *p*Bpa | 2 mM | 0.04 mM | 0.5 | |

[a]The compensating buffer is calculated according to **Table 13.2** except that the final intended concentration of $Mg^{2+}$ is now 8 mM.
[b]GSSG, oxidized Glutathion that is used to establish oxidative conditions during in vitro synthesis thereby allowing oxidative folding of the mature part of the TorA-PhoA precursor. Folding of pTorA-PhoA has been shown to be a prerequisite for a productive interaction of the signal peptide with TatC (28). This means that GSSG is a specific requirement of the substrate used in the experiment shown here and can therefore be omitted for other substrates.
[c]DNA used here is plasmid pET28aTorA-PhoAF15 (28).
[d]Depends on activity; use 5–10 U of a commercial enzyme.

tex and briefly spin to collect all liquid at the bottom of the tubes and incubate for additional 15 min on 37°C (*see* **Note 29**).

10. Add 45 µL of sample 2 to 45 µL 10% trichloracetic acid, mix, and let precipitate on ice for at least 30 min (*see* **Fig. 13.3**).
11. Spin the remaining samples 1, 3, and 4 and horizontally lay the closed tubes on ice. For UV irradiation of the samples, position UV lamp at the shortest possible distance right above the tubes (*see* **Note 30**). (UV radiation may

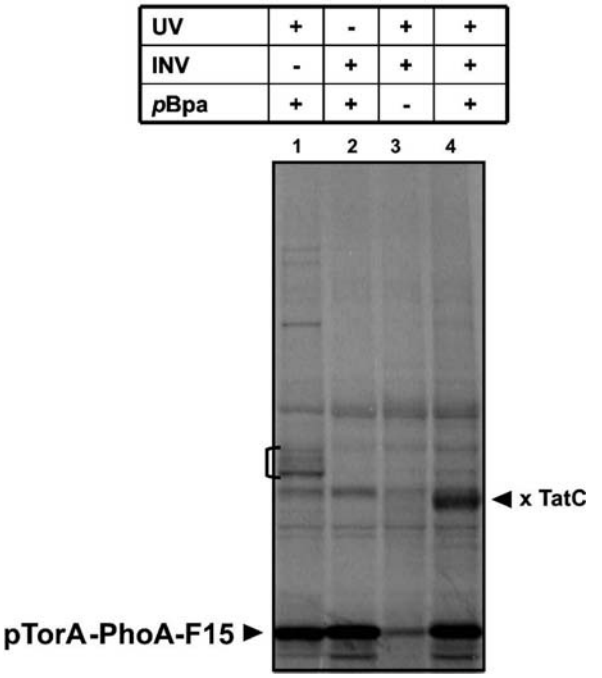


Fig. 13.4. In vitro synthesized pTorA-PhoA-F15 bearing *p*Bpa cross-links to TatC of inverted inner membrane vesicles (INV) from a TatABC-overproducing *E. coli* strain. Autoradiogram of the experiment described in the text (*see* **Section 3.3.3**). In vitro synthesis of pTorA-PhoA-F15 was performed using a coupled in vitro transcription-translation system from an *E. coli* strain transformed with plasmid pDULE. This plasmid encodes a *p*Bpa-specific amber suppressor tRNA together with a cognate, *p*Bpa-specific amino acyl-tRNA-synthetase. Efficient synthesis of pTorA-PhoA-F15 was obtained only if *p*Bpa was added (compare *lanes 3* to *lanes 1*, *2*, and *4*). Following synthesis, reactions were supplemented with inverted inner membrane vesicles (INV) prepared from strain BL21(DE3) pLysS p8737 as indicated. For activation of *p*Bpa incorporated in pTorA-PhoA-F15, samples were irradiated with UV-light (365 nm) and subsequently precipitated with trichloracetic acid. Samples were separated by SDS-PAGE and visualized by phosphorimaging ($\times$ TatC, cross-link between pTorA-PhoA-F15 and TatC). UV-irradiation yields several radioactively labelled products that are larger in size than pTorA-PhoA-F15. In the absence of INV, adducts around 80 kDa are obtained (*brackets*) that have previously been identified as cross-links to the chaperones FkpA and TorD (28). In the presence of INV, however, activation of *p*Bpa by UV-light leads to an adduct of approx 70 kDa that by immunoprecipitation was previously identified to result from an interaction of pTorA-PhoA-F15 with TatC (28). Note that cross-linking of the *p*Bpa-containing precursor with TatC only occurs upon UV-irradiation (compare *lanes 2* and *4*) and moreover does not occur for pTorA-PhoA-F15 resulting from read-through of the stop codon rather than from incorporation of *p*Bpa (*lane 3*).

cause damages to skin and eyes. Always wear gloves and UV-protecting glasses when handling the UV source).

12. Add 45 µL each of sample 1, 3, and 4 to 45 µL 10% trichloracetic acid, mix, and let it precipitate on ice for at least 30 min (*see* **Fig. 13.3**).

13. Add 30μL PAGE-loading buffer to each pellet and shake vigorously at room temperature to dissolve it completely. The colour of the loading buffer should remain dark blue. If it changes to yellow, add a few microliter of 1 M Tris base to neutralize residual trichloroacetic acid. Incubate samples at 37°C for 10 min (*see* **Note 31**) and analyse by SDS-PAGE and autoradiography. The results from this experiment are shown in **Fig. 13.4**.

## 4. Notes

1. Media and solutions are prepared using deionized water.
2. Tetracyclin is used here for the growth of *E. coli* strains transformed with plasmid pDULE-*p*Bpa (19) encoding the orthogonal pair of an amber suppressor tRNA, which specifically accepts the photo-reactive derivative of Phe, *p*Bpa, as well as its cognate pBpa-specific amino acyl-tRNA synthetase (17). We have successfully used the alternative plasmid pSup-BpaRS-6TRN(D286R) (20).
3. Stock solutions are usually freed of microorganisms and particles by filtration through 0.22 μm mixed cellulose ester filters (Millipore). Solutions 4–7, 10, and 14 are also required for the in vitro transcription/translation reaction (*see* **Section 2.3.1**).
4. Alternatively use Pefabloc SC (Roche), a water-soluble inhibitor of serine proteases at a final concentration of 0.5 mg/mL.
5. Use gloves to touch the dialysis tubing. Perform the following treatment before use: boil the dialysis tubing in 1 L 2% $NaHCO_3$, 1 mM EDTA for 10 min. Rinse the dialysis tubing with water and boil it again in 1 L water for 10 min. Store dialysis tubing in water at 4°C.
6. To obtain sufficient synthesis in vitro, the gene of interest preferably should be under the control of the T7 promoter. We have successfully used vectors such as pKSM717 (21) and pET derivatives.
7. To minimize sterical perturbances due to the incorporation of the bulky and hydrophobic site chain of *p*Bpa, preferentially replace amino acids exhibiting similar properties, such as Trp, Phe, Tyr, Val, and Leu.
8. If the efficiency of synthesis unexpectedly drops, it often can be overcome by preparing fresh stocks of spermidine, DTT, and phosphoenol pyruvate.

9. Polyethylene glycol, 18 amino acids, DTT, NTP stock, phosphoenol pyruvate, and creatine phosphate can be combined according to the ratios indicated in **Table 13.1** and stored in 100- to 200-μL aliquots at −20°C. This should be done only after proof has been obtained that the individual solutions allow efficient protein synthesis in vitro.
10. Although *p*Bpa is considered to be stable at ambient light (22), *p*Bpa stocks, as well as every *p*Bpa-containing sample in subsequent steps should be protected from light by covering with aluminium foil.
11. *E. coli* strains suitable for the preparation of S-135 are MC4100 (23) and Top10 (Invitrogen, Carlsbad, CA, USA). Other strains can likely be used as long as they do not contain an endogenous amber suppressor tRNA. DH10B (Invitrogen), for example, has been used for *p*Bpa-specific photo-cross-linking of proteins in vivo (19).
12. Compared to the starter culture the concentration of tetracyclin is reduced in order to maximize protein expression (19).
13. Fast resuspension is achieved by repeatedly forcing the cell suspension through the medium-bore opening of a ball-equipped glass pipet harbouring a sufficiently large reservoir.
14. The best result is obtained by passing the cell suspension through the French pressure cell at a speed that allows a dropwise efflux. This requires more than one passage as the released DNA first causes high viscosity until it becomes fragmented by the applied shear forces.
15. Freeze the S-30 immediately in liquid nitrogen and store at −80°C or continue with the next step.
16. Use a volume ratio of S-30 to dialysis buffer of approx 1:100. One of the three steps can conveniently also be done overnight. After dialysis, the S-30 can be quick-frozen in liquid nitrogen and stored at −80°C.
17. High-speed centrifugation resulting in an S-135 extract is required to remove all membrane vesicles from the S-30 extract. High speed centrifugation also removes remaining polysomes from the extract.
18. Note that the time of spin and amount of supernatant withdrawn will have an influence on the performance of the S-135, the designation of which is an operational term rather than reflecting the actual g-force. Recovery of too much supernatant might still result in a contamination with endogenous membranes, whereas too little supernatant bears the risk of a shortage of monosomes. In the

latter case try to reduce time of ultracentrifugation when preparing the S-135 or add separately isolated ribosomes. Low translation activity of an S-135 preparation can also result from residual cold methionine added for the readout of endogenous polysomes. This is effectively removed by repeated passages of the S-135 through an Amicon ultra centrifugal filter unit (Millipore, molecular weight cut off of 10,000 Da), each time replacing the filtrate by fresh S-30 buffer.

19. Do not thaw and freeze the S-135 more than twice.
20. Efficient cross-linking of twin-arginine-containing precursor proteins to INV requires the preparation of INV from strains that overproduce the TatABC proteins, such as BL21(DE3) pLysS p8737TatABCD (24) or DADE (MC4100, Δ*tatABCD*Δ*tatE*) (25) transformed with plasmids pRep4 and pQE60-TatABC (16). In the BL21 derivative, in which *tatABC* is under T7 promoter control, IPTG induces expression of T7 RNA polymerase, whereas in the other strain, IPTG directly enhances expression of *tatABC* from the *lac* promoter. High levels of the TatABC proteins in the vesicles prepared from these strains are verified via Western blotting.
21. Cells destined for the preparation of INV must not be frozen before breakage in the French press. If necessary cell pellets can be kept on ice overnight.
22. If the protocol is to be directly continued beyond this step, prepare sucrose gradients during this 2-h centrifugation period.
23. For determining the absorbance of the vesicle suspension at 280 nm, prepare a 1:100 dilution in 2% SDS. With an absorbance of 30 or more, usually 1–2 μL of INV are sufficient to observe cross-links of a Tat precursor protein to the TatABC proteins in a 25-μL reaction.
24. Do not freeze and thaw INV more than two or three times.
25. To avoid contamination with proteases and RNases, always wear gloves and preferentially use sterile disposable reaction tubes and pipet tips.
26. With every new preparation of S-135 it is necessary to re-adjust the reaction conditions. The variable with the strongest impact on expression efficiency is the concentration of $Mg^{2+}$ which even needs to be optimized for each particular DNA template. Sometimes inclusion of 8 mM putrescin into the reaction helps to improve expression. In this case the final $Mg^{2+}$ concentration is usually lowered by about 3 mM and that of phosphoenol pyruvate by 6 mM.

If in vitro expression remains unsatisfactory, try to vary the amount of S-135 in the range of 2–4μL. Calculate and prepare a new CB for each experiment.

27. Incubation at 37°C is routinely used. In some cases (e.g. INV derived from *cs* mutants) it is necessary and possible to synthesize proteins also at lower temperatures.
28. Precipitation with trichloroacetic acid can be extended to an overnight incubation.
29. Incubation with INV enables the in vitro synthesized precursor to interact with the TatC protein. Omission of an energy-regenerating system largely prevents the $H^+$-gradient-dependent translocation of the precursor by the Tat translocase and thereby guarantees a prolonged interaction of the precursor with TatC, which is part of the receptor complex recognizing the precursor at the initial step of the translocational process (15, 26, 27).
30. The UV-lamp used here emits light of 365 nm. This wavelength is suitable for activation of *p*Bpa (activation at 350–360 nm (22)). Since we determined that approx 65–70% of the activating light is absorbed by the wall of the reaction tube, the time of irradiation might be shortened by placing the tube vertically on ice with the lid open. Using the same setup of irradiation, the sample size can be varied considerably, as long as the ratio between the irradiated surface of the sample and its volume is kept constant (19).
31. Because TatC is a largely hydrophobic membrane protein, denaturation should not be performed by boiling in SDS in order to avoid a smeary appearance on SDS-PAGE.

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