Chapter 11

Membrane Protein Expression in Cell-Free Systems

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Abstract

Cell-free expression has emerged as a promising tool for the fast and efficient production of membrane proteins. The rapidly growing number of successfully produced targets in combination with the continuous development of new applications significantly promotes the distribution of this technology. Membrane protein synthesis by cell-free expression does not appear to be restricted by origin, size or topology of the target, and its global application is therefore a highly valuable characteristic. The technology is relatively fast to establish in standard biochemical labs, and it does not require expensive equipment. Moreover, it enables the production of membrane proteins in completely new modes, like the direct translation into detergent micelles, which is not possible with any other expression system. In this protocol, we focus on the currently most efficient cell-free expression system for membrane proteins based on *Escherichia coli* extracts.

Key words: Cell extracts, cell-free expression system, coupled transcription/translation, detergents, integral membrane protein, solubilization

1. Introduction

Cell-free (CF) expression systems have emerged as efficient and versatile tools for the production of even complex integral membrane proteins (MPs) (1–3). The CF approach mimics the natural cytoplasmic cell environment yet remains independent from the requirements and sensitivity of living cells. It thus overcomes general limitations known from conventional cellular expression systems, like toxic effects caused by membrane incorporation of recombinant MP, by incompatibility of MP function with cell physiology or by overloading of essential MP-targeting machineries. In addition, the option to synthesize MPs directly into artificial hydrophobic environments like detergent micelles makes CF

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expression strategies independent from complex and inefficient transport and translocation systems necessary for the localization of MPs into lipid membranes (4). Depending on the selected CF protocol and reaction setup, incorporation efficiencies of supplied amino acids into the synthesised protein of higher than 10% can be obtained, resulting in the production of several milligrams of protein per 1 mL of reaction (5, 6). Further beneficial characteristics of CF expression systems are small handling volumes of only few millilitres, short reaction times that usually finish within a day, high tolerance of the CF systems for a wide variety of substances like detergents or lipids and high efficiency in label incorporation combined with almost no background protein expression.

In contrast to conventional in vivo expression systems are the translation machineries of CF systems: open, accessible and not enclosed by a membrane. This feature primarily determines the high versatility of CF expression systems, allowing the supplementation of compounds at any point of the reaction. Such target-specific additives could be bioactive substances like ligands, substrates or inhibitors, but even protein components serving as chaperones, subunits or coenzymes could be considered. It should therefore be emphasised that reaction protocols can easily be modified by the supplementation of potentially beneficial additives according to specific requirements of a given MP target. Furthermore, efficient labelling of an expressed MP with, for example, seleno-methionine, ^{15}N- or ^{13}C-labelled amino acids or fluorescence-enhanced amino acids can simply be done by replacement of the desired amino acids in the reaction mixture (RM) with their labelled derivatives (7–9).

Different CF expression modes, extract sources and reaction setups for the CF expression of MPs have been established, and the optimization and modification of these protocols is still a highly dynamic field. MPs can be CF produced as precipitate (P-CF) and solubilised in different detergents after expression (see Fig. 11.1a) (1, 10). They can further be expressed directly in soluble modes by providing hydrophobic environments with detergents (D-CF), lipids (L-CF) or mixtures thereof (6, 11–14). The most frequently used extract sources are currently *Escherichia coli* cells, wheat germs and rabbit reticulocytes. Post-translational modifications other than disulphide bridge formation have not been reported from either *E. coli* or wheat germ systems (15). Some evidence of glycosylations in the less-productive rabbit reticulocyte extracts exists (16). Reaction setups could be one-compartment batch formats or the more complicated two-compartment continuous exchange cell-free (CECF) formats (see Fig. 11.1b). The more efficient CECF configuration contains a reservoir of low molecular weight precursors in a feeding mixture (FM) that is separated from the RM by a semi-permeable membrane (17, 18). This ensures the exchange of substrates and

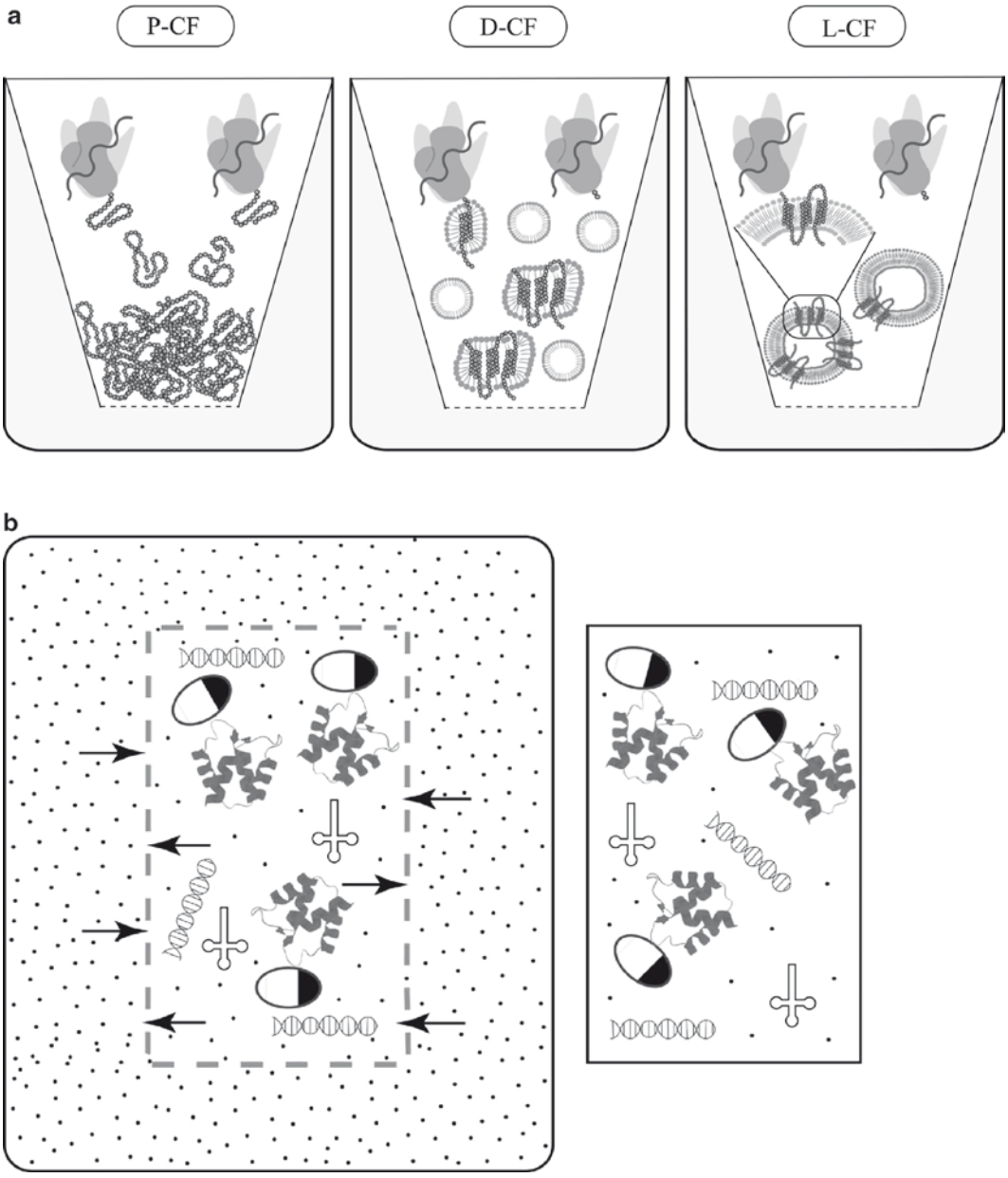

Fig. 11.1. Modes and systems for the CF expression of MPs. (**a**) Expression modes for the CF production of MPs. In the precipitate mode (P-CF), no hydrophobic environments are provided, and the MPs precipitate after translation. In the detergent-based mode (D-CF), the synthesised MPs can stay soluble by insertion into the provided detergent micelles. In the lipid-based mode (L-CF), the synthesised MPs have the possibility to integrate into supplemented liposomes. (**b**) CF expression in the CECF (***left***) and in the batch system (*right*). The two-compartment CECF system consists of a RM and an FM. Both are separated by a semi-permeable membrane, which provides optimal exchange of low molecular weight compounds. In the batch system, only a RM compartment is present.

by-products between the two compartments over a certain time period. resulting in higher production efficiency that can yield several milligrams of MP per 1 mL of RM. CECF reactions can be

scaled up to RM volumes of several millilitres without significant loss of efficiency. This chapter describes the basic protocols for the general CF production of MPs in the CECF configuration using *E. coli* extracts.

2. Materials

2.1. Preparation of T7 Polymerase

1. Bacterial strain, like *E. coli* BL21 (DE3) (Merck, Darmstadt, Germany).
2. Bactotryptone.
3. Chloroform.
4. Dialysis buffer: 10 m*M* K_2HPO_4/KH_2PO_4, pH 8.0, 10 m*M* NaCl, 0.5 m*M* ethylenediaminetetraacetic acid (EDTA), 5% glycerol and 1 m*M* 1,4-dithiotreitol (DTT).
5. Dialysis membrane, molecular weight cutoff (MWCO) 30 kDa.
6. Equilibration buffer: 30 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 50 m*M* NaCl, 5% glycerol and 10 m*M* β-mercaptoethanol.
7. 1*M* isopropyl β-d-1-thiogalactopyranoside (IPTG).
8. Phenol.
9. Q-Sepharose column.
10. Plasmid pAR1219 or another plasmid carrying the gene for T7 RNA polymerase (T7RNAP).
11. Resuspension buffer: 30 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 50 m*M* NaCl, 5% glycerol and 10 m*M* β-mercaptoethanol.
12. Sample concentrators, MWCO 30,000.
13. Sonicator or French press device.
14. 30% (w/v) streptomycin sulphate.
15. 5X transcription buffer: 200 m*M* Tris-HCl, pH 8.0, 60 m*M* $MgCl_2$, 25 m*M* DTT and 5 m*M* spermidine.
16. Yeast extract.

2.2. Preparation of E. coli S30 Extract

1. 10 L fermenter.
2. Centrifuge.
3. Photometer.
4. Dialysis membrane, MWCO 12–14 kDa.
5. 2*M* DTT.
6. 2xYTPG medium (per litre): 2.99 g KH_2PO_4, 6.97 g K_2HPO_4, 19.82 g glucose, 16 g tryptone, 10 g yeast extract, 5 g NaCl.

7. β-Mercaptoethanol.
8. Bacterial strains like *E. coli* A19 (*E. coli* Genetic Stock Center, New Haven, CT) or *E. coli* BL21 DE3 (Merck) or *E. coli* Rosetta (Merck) or *E. coli* BL21-Star (DE3) (Invitrogen, Carlsbad, CA) (see Note 1).
9. Bactotryptone.
10. French press cell disruption device or high-pressure cell homogenizer.
11. Liquid nitrogen.
12. Phenylmethanesulfonylfluoride (PMSF).
13. Tris-(hydroxymethyl)-aminomethan (Tris).
14. S30-A buffer: 10 m*M* Tris-acetate, pH 8.2, 14 m*M* magnesium acetate tetrahydrate [$Mg(OAc)_2$], 0.6 m*M* KCl, 6 m*M* β-mercaptoethanol (see Note 2).
15. S30-B buffer: 10 m*M* Tris-acetate, pH 8.2, 14 m*M* $Mg(OAc)_2$, 0.6 m*M* KCl, 1 m*M* DTT and 0.1 m*M* PMSF (see Note 2).
16. S30-C buffer: 10 m*M* Tris-acetate, pH 8.2, 14 m*M* $Mg(OAc)_2$, 0.6 m*M* potassium acetate (KOAc), 0.5 m*M* DTT (see Note 2).
17. Yeast extract.

2.3. E. coli CECF Expression

All solutions are prepared with Milli-Q water and kept at –20°C if not stated otherwise.

1. Reaction container (see Section 11.2.4. and Fig. 11.2).
2. Dialysis membrane, MWCO 12–14 kDa.
3. Slide-A-Lyzer (Pierce, Rockford, IL, USA), 10-kDa MWCO (see Note 3).
4. 50-mL glass vials.
5. Rolling device (Fröbel Labortechnik, Lindau, Germany) or temperature-controlled shaking incubator.
6. 500 m*M* DTT.
7. 1*M* acetyl phosphate lithium potassium salt (AcP), pH 7.0 adjusted with KOH.
8. 4 m*M* amino acid stocks (see Note 4).
9. 16.7 m*M* amino acid mix R, C, W, M, D and E (see Note 5).
10. 50-fold stock Complete® protease inhibitors cocktail with EDTA (Roche Diagnostics).
11. Detergents: Polyoxyethylene-(23)-laurylether (Brij-35), polyoxyethylene-(20)-cetyl-ether (Brij-58), polyoxyethylene-(20)-stearylether (Brij-78), digitonin (Sigma-Aldrich); n-dodecyl-β-d-maltoside (DDM) (Applichem Sigma-Aldrich); 1-myristoyl-2-hydroxy-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (LMPG) (Avanti lipids); Fos-Choline 12 (DPC) (Anatrace) (see Note 6).

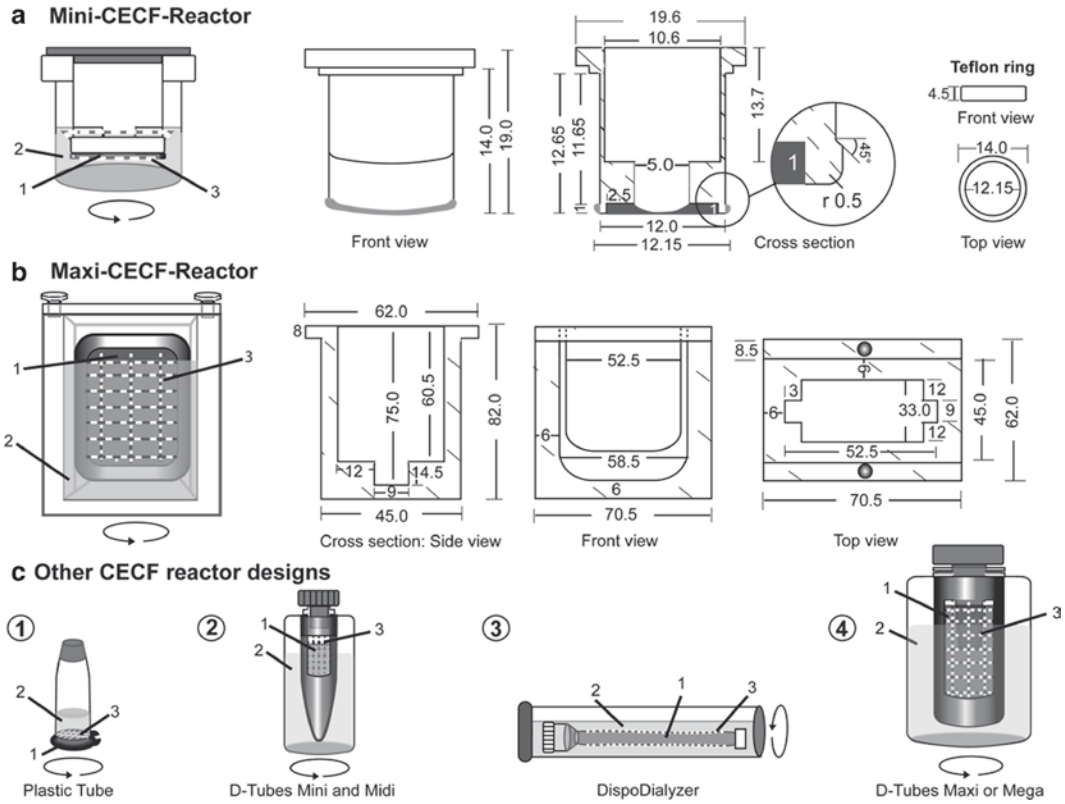

Fig. 11.2. Reaction containers suitable for CECF expression. (**a**) Dimensional sketch of CECF mini-reactors as described (19). The RM is kept in a flat chamber for optimal volume-to-surface ratio, which is highlighted in *dark* in the cross section. Volumes of 50–70 μL are commonly used. These containers are suitable for incubation in 24-well plates with the wells holding the appropriate volume of FM. (**b**) Dimensional sketch of CECF maxi-reactors. Commercially available Slide-A-Lyzers are used as containers for the RM; these are placed in a Plexiglas box that is specifically designed for a 1: 17 ratio of RM to FM (see Note 24). (**c1**) Standard 0.5-mL plastic reaction tube. The inner part of the cap holds the RM; the membrane is fixed between the cap and the tube. The bottom part of the tube is cut off, and the FM can then be filled into the tube. The tube is then sealed with Parafilm to prevent evaporation. Substance exchange between RM and FM can be improved by placing an appropriate magnetic bar in the RM to allow stirring during incubation. (**c2**) D-Tubes Mini and Midi. The RM is inside the reaction vial, which can be closed by a screw cap. For incubation, the D-tube has to be placed into a selected container of suitable size holding the FM. (**c3**) DispoDialyzer. The assembly is the same as explained for the analytical-scale D-tube devices. (**c4**) D-Tubes Maxi or Mega. **1** RM, **2** FM, **3** dialysis membrane.

12. 10 mg/mL folinic acid calcium salt.
13. 2.4*M* HEPES, pH 8.0, adjusted with KOH.
14. 1*M* $Mg(OAc)_2$.
15. 75-fold NTP mix: 90 m*M* adenosine 5′-triphosphate (ATP), 60 m*M* each guanosine 5′-triphosphate (GTP), cytosine 5′–triphosphate (CTP) and uridine 5′–triphosphate (UTP) (Roche Diagnostics), pH 7.0 adjusted with NaOH.
16. 1*M* phospho(enol)pyruvic acid monopotassium salt (PEP) (Sigma-Aldrich), pH 7.0 adjusted with KOH.

17. Plasmid vectors containing T7 regulatory elements, like pET (Merck) or pIVEX (Roche Diagnostics).
18. Plasmid DNA purification kits (e.g., Macherey and Nagel).
19. 40% (w/v) polyethylene glycol 8000 (PEG8000).
20. 4*M* KOAc.
21. 10 mg/mL pyruvate kinase (Roche Diagnostics).
22. 40 U/μL RiboLock™ RNase (ribonuclease) inhibitor (Fermentas) (see Note 7).
23. S30-C buffer without DTT (see Note 2).
24. S30 extract (see Section 11.3.1.3.).
25. 10% (w/v) sodium azide (NaN_3). Store at room temperature (RT).
26. 400 U/μL T7-RNA polymerase (see Section 11.3.1.2.) (see Note 8).
27. 40 mg/mL total transfer RNA (tRNA) *E. coli* (Roche Diagnostics) (see Note 9).

2.4. Reaction Container

1. Home-made container:

CECF mini-reactor for 50–70 μL RM, composed of Plexiglas with a Teflon ring (see Fig. 11.2a) (19).

CECF maxi-reactor for 1–3 mL RM, composed of Plexiglas (see Fig. 11.2b) (14).

2. Commercial container (see Fig. 11.2c):

D-Tubes 12- to 14-kDa MWCO (Merck) for analytical and preparative scale. DispoDialyzer (Spectrum, Rancho Dominguez, CA, USA) with a MWCO of 25 kDa composed of regenerated cellulose for preparative scale.

3. Methods

3.1. Preparation of Basic Reaction Components

3.1.1. Template Design for E. coli Extract-Based CF Expression

The T7RNAP can be used for DNA template transcription in *E. coli* systems if specific regulatory sequences have been attached to the target sequences (see Fig. 11.3). Key elements for *E. coli* CF systems are T7-promotor, Epsilon-enhancer of T7 phage gene g10, the ribosome -binding site and a T7-terminator region. It is also recommended to attach small purification or detection tags like, for example, a poly(His)n-tag, Strep-tag or T7-tag to the N- or C-terminus of the MP. In particular, codon-optimized N-terminal tags could generally enhance the expression levels (3, 11, 14). Standard expression vectors like pIVEX or pET

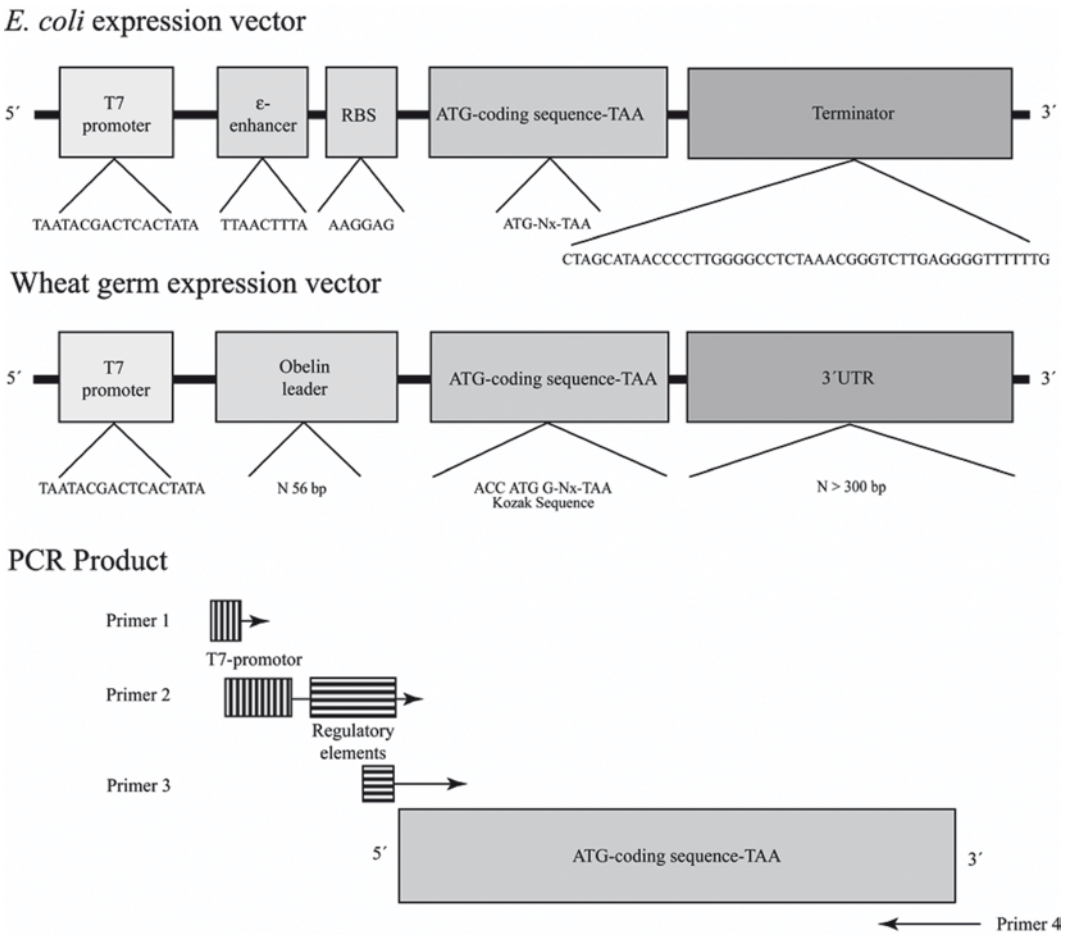

Fig. 11.3. DNA template construction for the CF expression of MPs with the T7RNAP in *E. coli* extracts. Optimal regulatory elements controlling the transcription of the target sequence comprise the T7-promotor, Epsilon-enhancer, a ribosom-binding site and the T7-terminator. PCR DNA templates can be generated by, for example, the split-primer PCR. This approach requires four primers as illustrated. First, a PCR product is generated with equal amounts of primers 3 and 4 (10 n*M* each). This product is then used as template in a second PCR reaction with a 100-fold excess of primers 1 and 4 with respect to primer 2 (100 to 1 n*M*).

derivatives can be used. DNA templates can be provided either as plasmids or as polymerase chain reaction (PCR) products. Linear PCR templates containing all required T7-promoter sequences can be generated by the split primer method using several overlapping primers (see Fig. 11.3) (20). The quality of DNA templates is crucial for the success of CF reactions. The template should be free of salts and residual RNA. If RNase was used during plasmid preparation, phenol/chloroform deproteinisation should be performed. In addition, relatively high concentrations of approximately 0.2 mg/mL are necessary, and the DNA should be resolved in water (see Note 10).

3.1.2. T7-RNA Polymerase Preparation

T7RNAP is needed in high amounts and in high concentrations, and it can efficiently be prepared by heterologous overproduction in BL21 (DE3) cells.

1. Take a fresh overnight culture of BL21 (DE3) × pAR1219, carrying the gene for the T7RNA polymerase (21) and inoculate 1 L LB medium in a ratio of 1:100 in a culture flask.
2. Incubate on a shaker at 37°C until an OD_{600} of 0.6–0.8 and induce expression with 1 m*M* IPTG.
3. After 5 h of incubation at 37°C, harvest the cells for 15 min at 4,500*g* and 4°C.
4. Resuspend the pellet in 30 mL resuspension buffer (see Section 11.2.1.).
5. Disrupt the cells with a French press cell-disrupting device at 20,000 psi or by sonication.
6. Centrifuge for 30 min at 20,000*g* and 4°C.
7. Precipitate nucleic acids with 4% streptomycin sulphate for 5 min on ice and centrifuge for 30 min at 20,000*g* and 4°C.
8. Apply the supernatant on a 40-mL Q-Sepharose column equilibrated with 2 CV equilibration buffer (see Section 11.2.1.).
9. Remove unbound proteins by washing with equilibration buffer (see Section 11.2.1.) at a flow rate of 4 mL/min.
10. Elute bound proteins with a gradient from 50 to 500 m*M* NaCl at 3 mL/min.
11. Pool the purest fractions containing T7RNAP and dialyse overnight against the dialysis buffer (see Section 11.2.1.).
12. Concentrate T7RNAP to 1–4 mg/mL by ultrafiltration. Adjust to 10% glycerol to avoid precipitation of the T7RNAP during the concentration procedure.
13. Store T7RNAP in aliquots in the dialysis buffer adjusted to 50% glycerol at –70°C up to 1 year.

The catalytic activity of purified T7RNAP can be analysed by in vitro transcription and be compared to the activity of a commercially obtained polymerase. In that case, the protocol has to be continued with the following steps: As template, a plasmid containing a reading frame under control of the T7-promoter has to be used. A template messenger RNA (mRNA) of defined size is produced by runoff transcription after linearising the plasmid by restriction at the 3′ end of the reading frame.

14. Purify the linearised plasmid from restriction enzymes by phenol/chloroform extraction.
15. Combine 50 ng of linearised plasmid with 0.1 U/µL RiboLock, 2 m*M* dNTPs and transcription buffer (see Section 11.2.1.).
16. Add different concentrations of the purified T7RNAP samples.

17. Incubate the reactions for 2 h at 37°C and stop by adding 2 m*M* EDTA.
18. Analyse and compare the intensity of the synthesized RNAs on agarose gels.

3.1.3. Preparation of E. coli Extracts

The protocol described is principally based on Zubay's procedure for preparation of S30 extracts (22), but it utilizes a modified runoff step.

First day

1. Inoculate 100 mL LB media with the selected strain and incubate with shaking at 37°C overnight.
2. Prepare the 2xYTPG media in the 10 L fermenter (see Note 11).
3. Chill 15 L of distilled water.
4. Prepare 50-fold stock solutions for S30-A, -B and -C buffer, without β-mercaptoethanol, DTT and PMSF, sterile filter and store them at 4°C. Prepare also a 4*M* NaCl solution for the dissociation step.
5. Autoclave a funnel for filling the components into the fermenter.

Second day

6. Inoculate the 10 L fermenter with 2xYTPG medium in a ratio of 1:100 with the preculture (see Note 12).
7. Grow the cells at 37°C with 500 rpm stirring and high aeration until they reach mid-logarithmic phase (see Note 13).
8. Chill the cells within the fermenter rapidly to a temperature below 12°C. This step should not exceed 45 min. After this step, the cells have to be kept at 4°C.
9. Harvest the cells by centrifugation at 5,000*g* for 15 min at 4°C.
10. Resuspend the pellet with a glass stick in 300 mL precooled S30-A buffer and centrifuge for 15 min at 5,000*g* and 4°C. Repeat this step three times with the same buffer but extend the centrifugation time at the final step for 30 min.
11. Weigh the pellet and resuspend it in 110% (v/w) of precooled S30-B buffer. A 10-L fermenter should produce a pellet with a wet weight of approximately 100–150 g depending on the fermentation conditions (see Note 14).
12. Disrupt the cells once with a precooled French press device using a constant pressure of 20,000 psi or with a high-pressure homogenizer at 17,500 psi and a flow rate of 1–3 mL/min (see Note 15).
13. Centrifuge the disrupted cells at 30,000*g* for 30 min at 4°C. Take the upper non-turbid two-thirds of the supernatant and transfer it to fresh centrifuge tubes. Repeat the centrifugation

step with the supernatant and again take the upper two-thirds of the supernatant.

14. The supernatant has to be adjusted to a final concentration of 400 m*M* NaCl followed by incubation at 42°C for 45 min. This step is used to get rid of endogenous mRNA. The solution will become turbid after incubation (see Note 16).
15. Dialyze the turbid extract first against 100 volumes of pre-cooled S30-C buffer for 2 h at 4°C with gentle stirring using a dialysis membrane with a 12- to 14-kDa MWCO. Change buffer once and dialyse a second time overnight at 4°C.
    Third day
16. Centrifuge the extract at 30,000*g* for 30 min at 4°C.
17. Aliquot the extract into suitable volumes and freeze immediately in liquid nitrogen. A 10-L fermenter should yield approximately 100–150 mL of final S30 extract. The frozen extract can be stored at −80°C for several months.

3.2. CECF Reaction Setup

CF reactions are performed in analytical scales for optimization studies and in preparative scales for high-level production (see Note 17). For the reaction setup, it is necessary to develop first a pipetting scheme (e.g., in Excel). Table 11.1 gives an example for the stock concentrations and final volumes in a reaction of 70 μL using the *E. coli* system.

3.2.1. Reaction Container Setup

Choice of appropriate reaction containers is a critical issue that can affect yield and reproducibility of a reaction. A variety of home-made or commercially available containers can be considered for analytical- or preparative-scale CECF reactions (see Fig. 11.2). The ratio between RM and FM should be between 1:10 and 1:30 for the best compromise of yields and costs (see Note 18). D-Tube dialyzers are available in different sizes, starting from 10 μL up to several millilitres and can be used for small analytical-scale reactions as well as for preparative-scale reactions. DispoDialyzers are recommended for RM volumes of 0.5–5 mL. The commercial devices serve as the RM container and have to be placed into suitable containers (e.g., plastic tubes) that hold the desired FM volume. The general handling of the commercial devices should be done according to the manufacturer's recommendations.

Analytical-Scale CECF Reactions in Microcentrifuge Tubes (18)

1. Remove the upper tip from the bottom part of a standard 0.5-mL microcentrifuge tube (e.g., Eppendorf) (see Note 19). Cut off the hinge from the lid and use the inner part of the lid as the RM chamber and the tube for the FM.
2. Completely fill the lid with approximately 100 μL of RM.
3. Cover the lid with a disk of dialysis membrane approximately 8 mm in diameter and with the desired MWCO. Ensure that no air bubbles are trapped.

Table 11.1
Protocol for P-CF expression in CECF mini-reactors

Component (unit)	Stock concentration	Final[a] concentration	Added volume (µL)	Volumes allocated for	
				RM	FM
Master mix					
NaN_3 (%)	10	0.05	5.4	0.4	5
PEG 8000 (%)	40	2	53.5	3.5	50
KOAc (m*M*)[b]	4,000	150.8	40.3	2.6	37.7
$Mg(OAc)_2$ (m*M*)[b]	1,000	10.1	10.8	0.7	10.1
HEPES (*M*)	2.4	0.1	44.6	2.9	41.7
Complete® (-fold)	50	1	21.4	1.4	20
Folinic acid (mg/mL)	10	0.1	10.7	0.7	10
DTT (m*M*)	500	2	4.3	0.3	4
NTP-mix (-fold)	75	1	14.3	0.9	13.4
PEP (m*M*)	1,000	20	21.4	1.4	20
AcP (m*M*)	1,000	20	21.4	1.4	20
AA-mix (m*M*)	4	0.5	133.8	8.8	125[b]
RCWMDE-mix (m*M*)	16.7	1	64.2	4.2	60
Final volumes			**446.1**	**29.2**	**416.9**
Feeding mix					
Master mix	–		416.9		
AA-mix (m*M*)	4	1[c]	125		
S30-C buffer (%)	100	35	350		
Milli-Q water			Fill up to 1,000 µL		
Final volume FM			**1,000 µL**		
Reaction mix					
Master mix	-		29.2		
Pyruvate kinase (mg/mL)	10	0.04	0.3		
Plasmid DNA/PCR fragment (mg/mL)	0.2	0.015	5.3		
RiboLock (U/µL)	40	0.3	0.5		
T7RNAP (U/µL)	400	6	1.1		

(continued)

Table 11.1 (continued)

Component (unit)	Stock concentration	Final[a] concentration	Added volume (μL)	Volumes allocated for RM	Volumes allocated for FM
tRNA *Escherichia coli* (mg/mL)	40	0.5	0.9		
S30 extract (%)	100	35	24.5		
Milli-Q water			Fill up to 70 μL		
Final volume RM			**70 μL**		

[a]Final concentrations of low molecular weight compounds are equal in the RM and FM, except AA-mix
[b]The protocol is given for a final concentration of K^+ of 290 m*M* and Mg^{2+} of 15 m*M*, but both compounds are subject to optimization. Additional sources of K^+ and Mg^{2+} ions are HEPES, PEP, AcP and the S30 extract
[c]FM is supplemented with additional 0.5 m*M* of the AA-mix

4. Fix the dialysis membrane by carefully pressing down the tube on the lid. Prevent sliding or damage of the dialysis membrane (see Note 20).
5. Fill the FM into the tube through the bottom hole and cover the hole and the lid rim with Parafilm to prevent evaporation.
6. Place the tube, lid facing down, in a 48-well microplate and incubate on a shaker at 30°C (see Note 21).

Reactions in CECF Mini-Reactors (see Note 22)

1. Prepare pieces of dialysis membranes (2.5 × 2.5 cm) with the appropriate MWCO.
2. Fix the membranes with the Teflon ring to the reactor chamber (see Note 23).
3. Fill 50–70 μL of RM from the top into the reaction chamber.
4. Fill a cavity of a 24-well plate with 800–1,000 μL FM to give a final ratio of 1:14.
5. Place the reaction container into the cavity with the FM. Prevent air bubbles between the dialysis membrane and the FM.
6. Seal the reaction setup with Parafilm to prevent evaporation and incubate on a 30°C shaker.

Reactions in CECF Maxi-Reactors (see Note 22)

1. Home-made CECF maxi-reactors are combined with commercially available Slide-A-Lyzers, MWCO of 10 kDa, holding the RM. Fill the Slide-A-Lyzer with 1–3 mL of RM (see Note 3).

2. Add the FM in a RM-to-FM ratio of 1:17 in the container (see Note 24).
3. Put the Slide-A-Lyzer into the reactor. Close the container either with Parafilm or with a lid and incubate it in a 30°C shaker.

3.2.2. E. coli CECF Reaction Setup

1. Thaw all stock solutions; enzymes should be thawed on ice. Mix every stock thoroughly except extract and enzymes (see Note 25).
2. Prepare a master mix of all components that have identical molarities in FM and RM (see Table 11.1).
3. Mix and remove the required RM volume from the master mix to a separate tube and store it on ice.
4. Complete the FM with onefold S30-C buffer without DTT, additional 0.5 m*M* amino acid mixture and water and pre-incubate at 30°C.
5. Complete the RM with the enzymes, the DNA template and the S30 extract on ice. Carefully mix all components (see Note 25).
6. Fill the RM and the FM in a ratio of 1:14 or 1:17 in the selected reaction container and incubate overnight at 30°C with shaking or rolling for 16–24 h.
7. For MP analysis, centrifuge the RM after the reaction for 15 min at 18,000*g*. Analyse supernatant and pellet fractions separately by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting if appropriate. Resuspend the pellet in water or buffer equivalent to the volume of the RM and analyse 1–3 μL of the sample by SDS-PAGE (see Fig. 11.4).
8. To optimise the yield of expressed MPs, it is recommended to perform an initial Mg^{2+} and K^{+} screen (see Note 17) (14).

3.2.3. CF Expression Modes for MP Production

Different CF expression modes for the synthesis of MPs are possible (see Fig. 11.1a). In the absence of any hydrophobic environment in the precipitate CF expression mode (P-CF) MPs will become insoluble and form a precipitate. These precipitates appear to contain at least partly structured protein as they often can be solubilized in relatively mild detergents without denaturation/renaturation procedures (1, 10). If detergents or amphipols are added into the RM, the MPs can be inserted into micelles directly after or during translation. This detergent-based CF expression mode (D-CF) therefore produces soluble proteomicelles (6, 11, 12, 23). Alternatively, the synthesized MPs could become directly inserted into provided lipid environments in the lipid-based CF expression mode (L-CF). The lipids could be added in the form of defined liposomes (13), as complex mem-

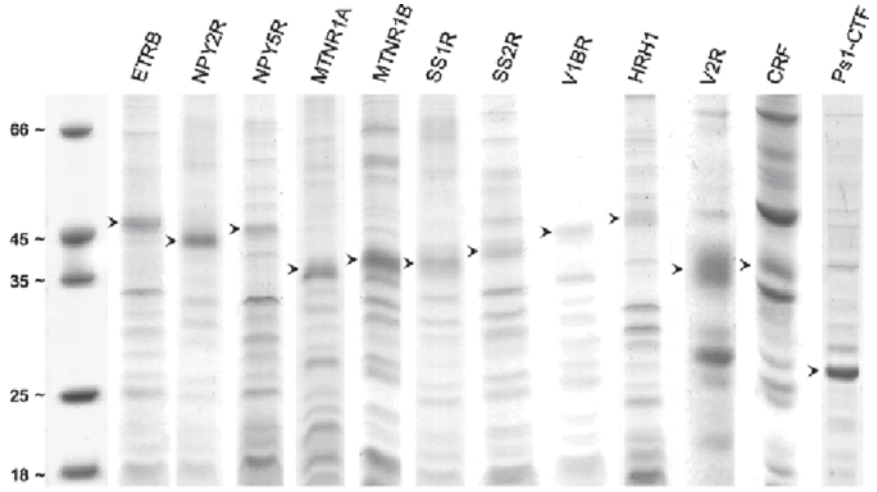

Fig. 11.4. Eukaryotic MPs expressed in the P-CF mode. Proteins were expressed in CECF mini-reactors, the RM was centrifuged at 18,000*g* for 10 min at 4°C, and the pellet was resuspended in a buffer volume equivalent to the RM volume. The following aliquots of these suspensions were mixed with loading buffer and separated by SDS-PAGE on a 12% gel and stained with Coomassie blue: *ETRB, NPY2R, NPY5R, MTNR1A, MTNR1B, SS1R, SS2R, V1BR, HRH1*: 3 µL; *V2R, CRF*: 2.5 µL; *PS1-CTF*: 4 µL. G protein-coupled receptors: ETRB (human endothelin B receptor); NPY2R and NPY5R (human neuropeptide Y2 and Y5 receptors, respectively); MTNR1A and MTNR1B (human melatonin 1A and 1B receptors); SS1R and SS2R (human somatostatin 1 and 2 receptors); V1BR (human vasopressin 1B receptor); HRH1 (human histamine 1 receptor); V2R (human vasopressin 2 receptor); CRF (rat corticotropin-releasing factor receptor); PS1-CTF: C-terminal fragment of human presenilin-1. The marker is given in kilodaltons; *arrows* indicate the expressed MPs.

brane vesicles (24), as giant liposomes out of egg yolk phosphatidylcholine (PC) and 1,2-dioleyl-sn-glycero-3-phosphocholine (DOPC) (25) or even as nanolipoprotein particles consisting of planar bilayers surrounded by a membrane scaffold protein (26). Mixtures of lipids and detergents provided as bicelles or mixed micelles might further facilitate the solubilization of expressed target MPs. Some residual *E. coli* lipids in amounts of approximately 100 µg/mL are furthermore still present in the S30 extract. The currently most established hydrophobic compounds that are suitable for the solubilization of MPs in the different CF expression modes are listed in Table 11.2. MP expression in the different L-CF modes is an emerging approach, and it is still too preliminary to recommend specific guidelines. Only details for the more established P-CF and D-CF modes are presented.

Solubilization of P-CF-Expressed MPs

P-CF mode reactions are in particular recommended for initial expression screens of new MP targets to evaluate the best individual reaction conditions. Yields of MPs are often significantly higher in the P-CF mode if compared with corresponding D-CF mode expressions, thus facilitating the expression monitoring by simple SDS-PAGE analysis (see Fig. 11.4). The basic reaction protocols established by P-CF expression screens can exactly be transferred as basic conditions for the expression in the D-CF mode.

1. Expression of MPs in the P-CF mode is done as described in Section 11.3.2.2. The RM should become turbid by the synthesised and precipitated MPs.
2. Harvest the MPs by centrifugation of the RM at 18,000*g* for 10 min at 4°C.
3. To reduce co-precipitated *E. coli* proteins, discard the supernatant and resuspend the pellet in appropriate solubilization buffer without detergent (e.g., 20 m*M* Na_2HPO_4, pH 7.5, 1 m*M* DTT), then centrifuge again for 10 min at 18,000*g* at 4°C. Repeat this washing step three times. More persistent co-precipitated proteins could be removed by washing in the presence of some detergents that are not effective in the solubilization of the target MP (e.g., Brij derivatives). Elaborated washing procedures could already result in relatively pure MP samples.
4. Resuspend the pellet in solubilization buffer with the appropriate detergent (e.g., 2% [w/v] LMPG) by pipetting up and down (see Note 26). Incubate for at least 30 min at 30–37°C with shaking (see Note 27).
5. Centrifuge once more for 10 min at 18,000*g* to pellet residual insoluble protein. Be careful about the centrifugation temperature because some detergents, like SDS and LMPG, will precipitate at lower temperatures. Take the supernatant for further studies.
6. Once solubilized, the detergent could be exchanged against more favourable ones after immobilizing the MP on a Ni^{2+} column by virtue of a terminal poly$(His)_n$-tag.

D-CF Expression of MPs

1. Prepare stock solutions of the selected detergents in distilled water in a concentration of at least ten times higher than the working concentration required for D-CF expression according to Table 11.2 (see Note 28). First, a screen with a variety of selected detergents or detergent combinations should be performed in analytical-scale reactions to identify the most suitable compound for the solubilization of the desired target MP.
2. Prepare a master mix like for the P-CF mode expression as given in Section 11.3.2.2. and add suitable amounts of detergent to reach the final concentrations given in Table 11.2. Subtract the added detergent volume from the water volume.
3. Continue as given in Section 11.3.2.2.
4. Successfully D-CF-expressed MPs will preferentially stay in the supernatant after centrifugation of the RM for 10 min at 18,000*g*. For the effective soluble expression, the MP should be found mainly in the soluble fraction in the SDS-PAGE. Since there are also many *E. coli* proteins in the soluble

Table 11.2
Additives suitable for the solubilization of CF-expressed MPs[a]

Supplement (mode)	CMC (m*M*)	Final conc. (%)	X CMC	Reference
Detergents (D-CF)				
Triton X-100	0.23	0.1 (0.2)	6.7 (13.4)	(6, 11)
DDM	0.12	0.1	15.0	(6)
DM	1.8	0.2	2.3	(6)
Digitonin	0.73	0.4	4.5	(6, 11)
Brij-35 (C12/23)	0.08	0.1	10.4	(6, 11)
Brij-58 (C16/20)	0.08	1.5	178.1	(6)
Brij-78 (C18/20)	0.05	1.0	189.0	(6)
Brij-98 (C18-1/20)	0.03	0.2	69.6	(6)
diC_8PC	0.22	0.1	8.9	(6)
Amphipols (D-CF)				
C6F-TAC	0.3	0.32–2.88	6.7–60	(23)
C8F-TAC	0.03	0.34–3.06	67–600	(23)
HF-TAC	0.44	0.36–3.2	4.5–40	(23)
Detergents (P-CF)				
LMPG	0.05	1–2	420–840	(6)
LPPG	n.a.	1–2	n.a.	(6)
DPC	0.9–1.5	1–2	19–38	(6)
SDS	9	1–2	4.2–8.4	(6)
Lipids (L-CF)				
Escherichia coli lipids		0.4		(1, 24)
DMPC		0.4		(13)
DOPC		0.4		(13)

[a]The table lists a selection of detergents and lipids that typically provide good results for MP production in the D-CF, P-CF or L-CF modes. The indicated concentrations are recommended for initial expression experiments and might be subject to optimization

CMC critical micellar concentration; *Triton X-100* polyethylene glycol P-1,1,3,3-tetra-methyl-butylphenyl ether; *DDM* n-dodecyl-β-D-maltoside; *DM* n-decyl-β-D-maltoside; *Brij-35* polyoxyethylene-(23)-lauryl-ether; *Brij-58* polyoxyethylene-(20)-cetyl-ether; *Brij-78* polyoxyethylene-(20)-stearyl-ether; *Brij-98* polyoxyethylene-(20)-oleyl-ether; *C_6F-TAC* $C_6F_{13}C_2H_4$-*S*-poly[tris(hydroxymethyl)aminomethane]; *C_8F-TAC* $C_8F_{17}C_2H_4$-*S*-poly[tris-(hydroxylmethyl) aminomethane]; *HF-TAC* $C_2H_5C_6F_{12}C_2H_4$-*S*-poly[tris (hydroxyl-methyl)-aminomethane]; *diC_8PC* 1,2-dioctanoyl-sn-glycero-3-phosphocholine; *LMPG* 1-myristoyl-2-hydroxy-sn-glycerol-3-[phosphor-rac-(1-glycerol)]; *LPPG* 1-palmitoyl-2-hydroxy-sn-glycerol-3-[phosphor-rac-(1-glycerol)]; *DPC* dodecylphosphocholine; *SDS* sodium dodecyl sulphate; *DMPC* 1,2-dimyristoyl-sn-glycero-3-phosphocholine; *DOPC* 1,2-dioleyl-sn-glycero-3-phosphocholine

fraction, it depends on the expression level whether the target MP can be detected. It is recommended to verify protein expression by Western blotting or to analyse samples after purification (e.g., by affinity chromatography) (see Note 29).

5. Identified optimal expression conditions can be scaled up to 1- to 3-mL preparative-scale reactions in the CECF maxi-reactors (see Note 18).

4. Notes

1. In our hands, *E. coli* K-12 strain A19 [rna19 gdh A2 his95 relA1 spoT1 metB1] (27) produces the most efficient extracts. Its ribonuclease I deficiency helps to stabilize mRNA during transcription/translation. However, other *E. coli* strains like BL21 (DE3) (Merck) and derivatives could also produce an effective extract (28, 29). The additional overexpression of rare codon tRNAs could be advantageous for the expression of heterologous MPs.
2. Prepare a 50-fold stock solution of the S30 buffers without DTT, β-mercaptoethanol or PMSF, which can be stored at 4°C up to several months.
3. The Slide-A-Lyzers can be reused several times for expression of the same protein until the membrane starts to become clogged. Each corner of the Slide-A-Lyzers has one gasket. Always use the same gasket for filling or removing the RM and place the Slide-A-Lyzer into the CECF maxi-reactor with the side containing the used gasket upward to prevent leakage of the RM. It is recommended to vent the Slide-A-Lyzer during the filling procedure with a needle inserted into the gasket at the other edge of the same side.
4. Prepare for tyrosine a 20 m*M* stock solution and for the other 19 amino acids 100 m*M* stock solutions. L-Tryptophan has to be dissolved in 100 m*M* HEPES, pH 8.0, L-aspartic acid, L-cysteine, L-glutamic acid and L-methionine have to be dissolved in 100 m*M* HEPES, pH 7.4. All other amino acids are dissolved in distilled water. Sonication or heating to 60°C could improve solubility. However, some stocks (in particular W, D, E, N, C, Y) will not dissolve completely and have to be handled as suspensions. For the amino acid mixture, take 2 mL of each of the 100 m*M* amino acid stocks and 10 mL of the 20 m*M* L-tyrosine stock and fill up with distilled water to 50 mL. This results in a 4 m*M* stock concentration of each amino acid. The standard final amino acid concentration in

the CF reaction is 1 m*M*, while higher concentrations might increase the final protein yields in some cases.

5. The amino acids R, C, W, M, D and E are subjected to metabolic degradation during incubation. Increased amounts enhance MP production.
6. Only few frequently used detergents are listed. CF systems are highly tolerant of a wide variety of detergents. For more complete lists, please consult Table 11.2 or (6, 11, 12).
7. Some commercially available RNase inhibitors may contain energy consumptive impurities. Evaluation of the quality is therefore recommended when using new batches or suppliers.
8. It is also possible to use commercially available T7RNAP (e.g., Roche Diagnostics), but besides the high costs, the concentrations are usually too low, and additional concentration steps would be required.
9. Increased final tRNA concentrations could reduce premature termination products of targets with larger size, heterologous origin and codon usages very different from that of the *E. coli* expression background.
10. The volume of the RM is limited, and higher concentrations of the DNA template save space for further additives. Relatively high amounts of DNA template are required, and vectors with increased copy numbers like the pIVEX might be preferred, if possible.
11. Autoclave 2xYT medium and phosphate buffer separately to avoid precipitation. Sterilize glucose by filtration. Combine the solutions and add 1–2 mL antifoam before starting the fermentation.
12. Stir the medium vigorously for 5 min before adding the preculture to enrich it with oxygen.
13. Harvesting of the cells in the mid-logarithmic phase is important because rapidly growing cells contain more active ribosomes, and extracts are therefore more productive. Rapid cooling of the cells after fermentation will conserve them in this active state.
14. After this step, the pellet could be frozen in thin layers at –70°C and stored for few weeks. However, it is recommended to continue with the extract preparation without storage to achieve highest activities.
15. Cell disruption by sonication might reduce extract activity as a result of ribosome disintegration.
16. This procedure causes the dissociation of ribosomes from mRNA, which subsequently will become degraded by

endogenous RNases. Incubation time and temperature are important to ensure the optimal extract efficiency while excessive inactivation of essential proteins has to be prevented.

17. For every new MP target expression optimization screens in the analytical scale for key compounds like Mg^{2+} and K^+ ions are highly recommended to achieve best results. With *E. coli* extracts and with the described protocols, Mg^{2+} concentrations between 13 and 17 m*M* and K^+ concentrations between 270 and 310 m*M* are usually optimal.
18. The exchange area of the membrane becomes more and more limiting with increased RM volumes, resulting in increasingly lower yields. Dialysis membranes with a relatively wide range of pore sizes between 10 and 50 kDa can be used. The choice of the MWCO also depends on the size of the expressed MP, which preferentially should remain in the RM. Membranes consisting of regenerated cellulose should be preferred.
19. The inner part of the lid fits approximately 100 µL of RM, and the tube fits 0.5 mL of FM, resulting in a somehow sub-optimal RM: FM ratio of 1:5. Alternatively, the RM volume could be decreased to 50 µL by addition of few glass beads into the lid chamber.
20. Damage of the membrane, as often observed when using larger 1.5-mL microcentrifuge tubes, will cause leakage.
21. Alternatively, a small magnetic bar could be included in the RM chamber and the tube be incubated on a magnetic stirrer.
22. All reactors should be cleaned with distilled water after use, and they must be completely dry before use.
23. The reaction chamber is formed between the dialysis membrane and a 1-mm cavity at the bottom part of the CECF mini-reactor, which is formed by a corresponding rim as illustrated in Fig. 11.2a. Every time, use new dialysis membranes for the CECF mini-reactors. Make sure that the Teflon ring is correctly adjusted on the same level as the reaction chamber and that the dialysis membrane is completely planar without any crinkles at the bottom part, which serves as exchange area between RM and FM. First, place the Teflon ring on a piece of Parafilm, cover the ring with the membrane and then press the reactor over the ring until it stops. The dialysis membrane should be large enough that its ends extend over the Teflon ring to ensure that the device is tight and no leakage of the RM occurs. The Teflon ring should fit tightly to the reactor without membrane. Correct adjusting and absence of leakage could preliminary be monitored with, for example, dextran blue.
24. For the CECF maxi-reactors, it is important that the levels of RM and FM are identical when the Slide-A-Lyzer is inside

the container. Adjust the levels by removing some air from the RM compartment or by addition of extra FM.

25. Too vigorous mixing might denature proteins or ribosomes in the extract.
26. Sometimes, the solution becomes clear during the pipetting process, in particular in the presence of SDS and LMPG. If the sample stays turbid, the target MP could still be present in the soluble fraction while other impurities might remain in the insoluble fraction. Choice of buffer and pH value are subject of optimization and can be crucial for the resolubilisation efficiencies.
27. The solubilization efficiency of different detergents strongly depends on each particular MP. Different types of detergents at different concentrations and at different temperatures have to be tested. LMPG, LPPG, SDS and DPC are usually efficient and among the first choices.
28. Detergent stocks should be as highly concentrated as possible. Higher concentrated stocks of long-chain Brij derivatives become soluble by incubation at 65°C, and they can be stored frozen at –20°C. The stocks might become turbid again after thawing or extended storage for hours in non-frozen conditions. It is recommended always to screen different detergent concentrations by expression in the D-CF mode as the detergent tolerance is also somehow target protein dependent.
29. If using a Ni^{2+} purification column, first dilute your sample with buffer to avoid Ni^{2+} reduction by the 2 m*M* DTT in the RM. Purification directly after expression is recommended.

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