Cell-Free Expression of G Protein–Coupled Receptors

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The large-scale production of recombinant G protein–coupled receptors (GPCRs) is one of the major bottlenecks that hamper functional and structural studies of this important class of integral membrane proteins. Heterologous overexpression of GPCRs often results in low yields of active protein, usually due to a combination of several factors, such as low expression levels, protein insolubility, host cell toxicity, and the need to use harsh and often denaturing detergents (e.g., SDS, LDAO, OG, and DDM, among others) to extract the recombinant receptor from the host cell membrane. Many of these problematic issues are inherently linked to cell-based expression systems and can therefore be circumvented by the use of cell-free systems. In this unit, we provide a range of protocols for the production of GPCRs in a cell-free expression system. Using this system, we typically obtain GPCR expression levels of ~1 mg per ml of reaction mixture in the continuous-exchange configuration. Although the protocols in this unit have been optimized for the cell-free expression of GPCRs, they should provide a good starting point for the production of other classes of membrane proteins, such as ion channels, aquaporins, carrier proteins, membrane-bound enzymes, and even large molecular complexes.

Keywords: cell-free expression • *E. coli* extract • membrane protein • GPCR • detergent • lipid

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INTRODUCTION

G protein–coupled receptors (GPCRs) represent the largest family of membrane proteins in the human genome and the richest source of targets for the pharmaceutical industry. Given that the majority of GPCR-targeted drugs in clinical practice (which represent more than 30% of all drugs) exert their actions on only 30 of the 750 identified human GPCRs, there is significant scope for further drug discovery in this field. Despite their profound importance in human health and disease, functional and structural characterization of GPCRs has been limited. This is illustrated by the fact that GPCR structures currently account for less than 1% of all known high-resolution protein structures (Kouranov et al., 2006). This disparity reflects the challenges in preparing functional GPCRs in quantities sufficient for structural investigations. These difficulties are most notably related to their hydrophobic nature and to their low natural abundance. Except for rhodopsin, whose crystal structure was solved following its extraction from the retina (Hofmann et al., 2009), GPCRs do not exist in native tissues at sufficiently high levels to allow their

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purification in biochemically relevant amounts. As a consequence, the vast majority of GPCRs need to be overexpressed in an appropriate host cell system before they can be purified to homogeneity.

One major problem upon GPCR overexpression in cellular systems is the efficient translocation of the recombinant membrane protein into the host cell membrane, which is based on complex trafficking signals and transport systems (Rached et al., 2005). Although relatively large amounts of membrane proteins might be produced in conventional living systems, often only a small proportion finally become integrated into the host cell membrane (Freigassner et al., 2009). In addition, overproduction of the heterologous membrane proteins often results in overloading of cellular transport and post-translational processing systems, which may cause further toxic effects (Lin and Guidotti, 2009). A second major challenge in recombinant GPCR production is the isolation of the expressed receptors from their native membrane environment, which often requires the use of harsh detergents (Lin and Guidotti, 2009). The choice of detergent is critical, as membrane proteins are very unstable and are readily denatured by detergent (Seddon et al., 2004; Lacapere et al., 2007).

Cell-free expression offers a unique opportunity to circumvent the above-mentioned bottlenecks that are inevitably linked to the recombinant production of membrane proteins. Cell-free expression systems are based on gene expression in the presence of cell extracts that provide the cellular components necessary for protein synthesis, such as ribosomes and initiation, elongation and termination factors (Rosenblum and Cooperman, 2014). The inherent open nature of cell-free systems allows the addition of almost any compound that might be beneficial for the folding or the stabilization of the recombinant protein, such as cofactors, protease inhibitors, stabilizing ligands, or inhibitors. In particular, the addition of (mild) detergents or even lipids in the form of liposomes or nanodiscs offers the possibility to synthesize membrane proteins directly into a well-defined hydrophobic environment (Rajesh et al., 2011; Bernhard and Tozawa, 2013; Zheng et al., 2014). Such a strategy not only prevents the aggregation of freshly synthesized membrane proteins, but also eliminates the often problematic detergent extraction step that is necessary to isolate the target protein from the host cell membrane. This approach, which is unique for the cell-free technique and not possible with any other expression system, offers the opportunity to specifically address problems with host cell toxicity, protein solubility, or instability (e.g., proteolysis).

In this unit, we provide methods for the recombinant production of GPCRs in an *E. coli* cell-free expression system. Support Protocols 1 and 2 describe how to prepare *E. coli* cell extracts for highly productive cell-free protein expression. In Basic Protocol 1, we show how the cell-free reaction parameters can be optimized using the green fluorescent protein (GFP) as a reporter. Because appropriate DNA template design is crucial to successful cell-free expression, possible strategies for the design of cell-free expression constructs are discussed in Basic Protocol 2. Basic Protocols 3 and 4 describe a method for the cell-free expression of membrane proteins in the absence of membrane mimetics (precipitate mode). Detailed reaction schemes for cell-free protein production on the analytical (batch configuration; Basic Protocol 3) and preparative scale (continuous-exchange configuration; Basic Protocol 4) are provided. Basic Protocol 5 provides procedures for the cell-free production of soluble membrane proteins in the presence of membrane mimetics such as detergent micelles or amphipathic polymers.

Although the protocols in this unit have been optimized for the cell-free expression of GPCRs, they should provide a good starting point for the production of other classes of membrane proteins. Examples of other types of membrane proteins that have already been successfully produced in *E. coli* cell-free expression systems include ion channels

(Deniaud et al., 2010; Rath et al., 2011), aquaporins (Muller-Lucks et al., 2013; Zhang et al., 2014), carrier proteins (Blesneac et al., 2012), membrane-bound enzymes (Boland et al., 2014), and even large molecular complexes (Matthies et al., 2011).

EXTRACT TESTING AND OPTIMIZATION OF CELL-FREE REACTION CONDITIONS

BASIC PROTOCOL 1

The optimal expression conditions vary slightly for each batch of extract, with Mg^{2+} and K^+ concentrations being the most sensitive. Therefore, the optimal Mg^{2+} and K^+ concentration should be determined for each new batch of S12 and S12-T7 extract and for each new protein target. Reporter proteins such as Green Fluorescent Protein are excellent tools for the rapid monitoring and quantification of cell-free reactions and for the optimization of cell-free reaction conditions. This basic protocol describes the setup of a correlated Mg^{2+}/K^+ optimization screen in analytical-scale reactions (70 µl) using free, wild-type GFP as a reporter protein. A similar experimental setup can be used to optimize other expression parameters, such as the concentration of plasmid DNA, the concentration and ratio of the S12 and S12-T7 extracts, or the expression temperature.

Alternatively, the target GPCR can be expressed as a C-terminal GFP fusion protein. This not only enables the rapid quantification of expression levels (e.g., for construct optimization), but also permits monitoring of the folding process within the crude cell-free reaction mixture (Muller-Lucks et al., 2012).

Materials

E. coli S12 cell extract (see Support Protocol 1)
E. coli S12-T7 cell extract (see Support Protocol 2)
100 mg/ml NaN_3 (Sigma)
400 mg/ml PEG8000 (Sigma)
4 M potassium acetate (KOAc; Sigma)
2.4 M HEPES (Sigma; adjust to pH 8.0 with KOH)
50× Complete protease inhibitor cocktail without EDTA (Roche Diagnostics); prepare by dissolving ten tablets in 10 ml of Milli-Q water
10 mg/ml folinic acid calcium salt (Sigma)
NTP mix (33.3 mM ATP, 22.2 mM UTP, 22.2 mM GTP, 22.2 mM CTP; see recipe)
430 mM phospo(enol)pyruvic acid monopotassium salt (PEP; Sigma; adjust to pH 7.0 with KOH)
1 M acetyl phosphate lithium potassium salt (AcP; Sigma; adjust to pH 7.0 with KOH)
Amino acid mix (4 mM each; see recipe)
RCWMDE amino acid mix (16.7 mM each, see recipe)
1 M magnesium acetate tetrahydrate [$Mg(OAc)_2$; Sigma]
10 mg/ml pyruvate kinase (Roche Diagnostics)
40 U/µl Ribolock RNase (ribonuclease) inhibitor (Thermo Scientific)
40 mg/ml *E. coli* total transfer RNA (tRNA; Roche Diagnostics)
2.8 M potassium acetate (KOAc; anhydrous; Sigma)
200 ng/µl plasmid DNA encoding GFP or eGFP (standard *E. coli* expression vectors like pIVEX or pET derivatives can be used)

Rolling device or temperature-controlled shaking incubator
Black Corning Costar 384-well plate
Fluorescence 96-/384-well plate reader

NOTE: The cell-free transcription/translation procedure is quite sensitive to the presence of RNases. Therefore, gloves should be worn at all times while making up the solutions and setting up the reactions. Disposable sterile plasticware or autoclaved glassware

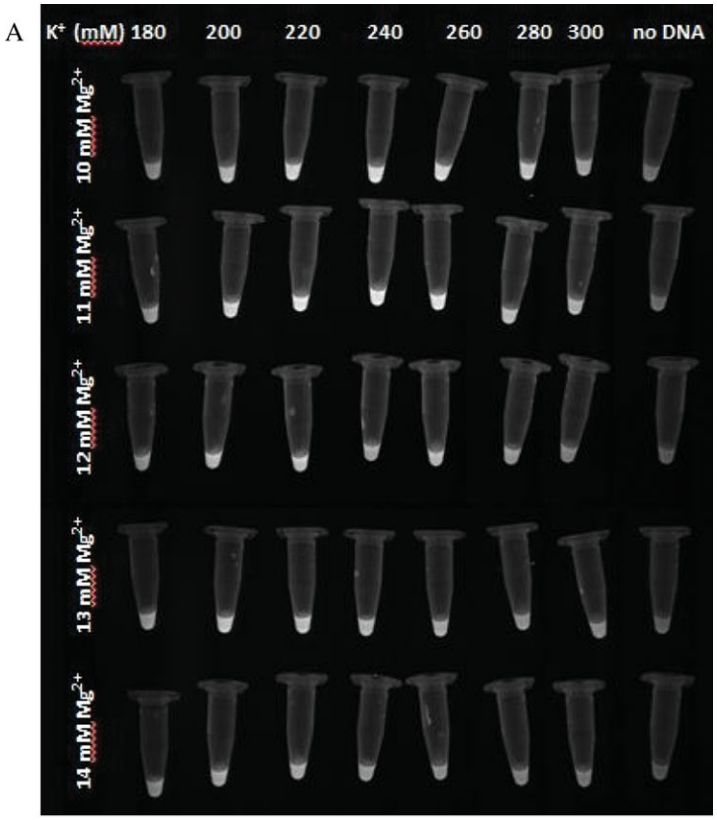

Figure 29.14.1 Example of a Mg^{2+}/K^{+} correlated optimization screen using GFP as reporter. GFP was expressed in 70-µl batch reactions in the presence of different Mg^{2+} (10 to 14 mM) and K^{+} (180 to 300 mM) concentrations, as described in Basic Protocol 1. After 5 hr incubation at 30°C, GFP fluorescence was visualized directly in the reaction tubes (Fig. 29.14.1A). The amount of expressed GFP was estimated by comparing the fluorescence signal of the cell-free samples to a standard curve of recombinant GFP (Fig. 29.14.1B).

should be used throughout. All chemicals are purchased as analytical-quality, RNase-free grade. Prepare all solutions with RNase-free Milli-Q water and keep at −20°C if not stated otherwise. Store all solutions in small working aliquots at −20°C to avoid repeated freeze-thaw cycles.

Setting up the reaction

For *E. coli* cell-free systems, the total Mg^{2+} ion concentrations typically range from 10 to 20 mM, while K^{+} can be added at high concentrations exceeding 200 mM. In this

Table 29.14.1 A Correlated Mg^{2+}/K^+ Ion Optimization Screen: Pipetting Scheme for the Preparation of Cell-Free Master Mixes Containing 180 mM K^+ and 10 to 14 mM Mg^{2+}

			180 mM K^+				
			10 mM Mg^{2+}	11 mM Mg^{2+}	12 mM Mg^{2+}	13 mM Mg^{2+}	14 mM Mg^{2+}
Component (unit)	Stock conc.	Final conc.	Volume (µl)				
NaN_3 (mg/ml)	100	0.5	3.2	3.2	3.2	3.2	3.2
PEG8000 (mg/ml)	400	20	31.5	31.5	31.5	31.5	31.5
K^+ acetate (mM)	4000	40	6.3	6.3	6.3	6.3	6.3
HEPES (M)	2.4	0.1	26.3	26.3	26.3	26.3	26.3
Complete protease inhibitor cocktail without EDTA (×)	50	1	12.6	12.6	12.6	12.6	12.6
Folinic acid calcium salt (mg/ml)	10	0.1	6.3	6.3	6.3	6.3	6.3
NTP mix (×)	27.78	1	22.7	22.7	22.7	22.7	22.7
Phospo(enol)pyruvic acid monopotassium salt (PEP) (mM)	430	20	29.3	29.3	29.3	29.3	29.3
Acetyl phosphate lithium potassium salt; AcP (mM)	1000	20	12.6	12.6	12.6	12.6	12.6
Amino acid mix (mM)	4	0.5	78.8	78.8	78.8	78.8	78.8
RCWMDE-mix (mM)	16.7	1	37.7	37.7	37.7	37.7	37.7
Magnesium acetate tetrahydrate [$Mg(OAc)_2$] (mM)	1000	10-14	3.2	3.8	4.5	5.1	5.7
Pyruvate kinase (mg/ml)	10	0.04	2.5	2.5	2.5	2.5	2.5
GFP template DNA (ng/µl)	200	15	47.3	47.3	47.3	47.3	47.3
Ribolock (U/µl)	40	0.3	4.7	4.7	4.7	4.7	4.7
tRNA *E. coli* (mg/ml)	40	0.5	7.9	7.9	7.9	7.9	7.9
S12 extract (%)	100	17.5	110.3	110.3	110.3	110.3	110.3
S12-T7 extract (%)	100	17.5	110.3	110.3	110.3	110.3	110.3
Milli-Q water (µl)			49.7	49.1	48.5	47.9	47.2
Total master mix volume (µl)			603.0	603.0	603.0	603.0	603.0

example, Mg^{2+} and K^+ concentrations are varied between 10 to 14 mM and 180 to 300 mM, respectively (Fig. 29.14.1).

1. Thaw an aliquot of the S12 and S12-T7 extracts and all the cell-free reaction components listed in Table 29.14.1.

   Enzymes and extracts should be thawed on ice.

2. Prepare five cell-free master mixtures (on ice) using the pipetting scheme described in Table 29.14.1.

   Each master mix contains 180 mM K^+, but different Mg^{2+} concentrations (10 to 14 mM).

Table 29.14.2 Correlated Mg^{2+}/K^+ Ion Optimization Screen: Pipetting Scheme for the Preparation of Cell-Free Reactions with Varying Concentrations of K^+ (180 to 300 mM) and Mg^{2+} (10 to 14 mM)

Final total [K^+] (mM)	180	200	220	240	260	280	300
µl of master mix (180 mM K^+; 10 mM Mg^{2+})	67	67	67	67	67	67	67
µl of KOAc (2.8 M)	0	0.5	1	1.5	2	2.5	3
µl Milli-Q water	3	2.5	2	1.5	1	0.5	0
Total reaction mix volume (µl)	70	70	70	70	70	70	70
Final total [K^+] (mM)	180	200	220	240	260	280	300
µl of master mix (180 mM K^+; 11 mM Mg^{2+})	67	67	67	67	67	67	67
µl of KOAc (2.8 M)	0	0.5	1	1.5	2	2.5	3
µl Milli-Q water	3	2.5	2	1.5	1	0.5	0
Total reaction mix volume (µl)	70	70	70	70	70	70	70
Final total [K^+] (mM)	180	200	220	240	260	280	300
µl of master mix (180 mM K^+; 12 mM Mg^{2+})	67	67	67	67	67	67	67
µl of KOAc (2.8 M)	0	0.5	1	1.5	2	2.5	3
µl Milli-Q water	3	2.5	2	1.5	1	0.5	0
Total reaction mix volume (µl)	70	70	70	70	70	70	70
Final total [K^+] (mM)	180	200	220	240	260	280	300
µl of master mix (180 mM K^+; 13 mM Mg^{2+})	67	67	67	67	67	67	67
µl of KOAc (2.8 M)	0	0.5	1	1.5	2	2.5	3
µl Milli-Q water	3	2.5	2	1.5	1	0.5	0
Total reaction mix volume (µl)	70	70	70	70	70	70	70
Final total [K^+] (mM)	180	200	220	240	260	280	300
µl of master mix (180 mM K^+; 14 mM Mg^{2+})	67	67	67	67	67	67	67
µl of KOAc (2.8 M)	0	0.5	1	1.5	2	2.5	3
µl Milli-Q water	3	2.5	2	1.5	1	0.5	0
Total reaction mix volume (µl)	70	70	70	70	70	70	70

Note that 4.9 mM of the total Mg^{2+} concentration is contributed by the S12 and S12-T7 extracts and that ~140 mM K^+ is brought in by other reaction components (e.g., AcP, PEP, KOH, S12 extract). Magnesium and potassium acetate are added to the cell-free master mixtures to obtain the desired final Mg^{2+} and K^+ concentrations.

3. Combine the calculated volumes of the master mixtures, a 2.8 M KOAc stock solution, and Milli-Q water as illustrated in Table 29.14.2.
4. Incubate the microcentrifuge tubes containing the cell-free reactions for 3 to 5 hr with gentle shaking or rolling at 30°C.
5. Transfer 30 µl of each reaction to a black-walled 384-well plate and measure GFP fluorescence (λ_{exc} 485 nm; λ_{em} 535 nm) in a plate reader.

   Optionally, prepare a GFP standard curve (ranging from 0 to 1000 µg/ml) using recombinant purified GFP to estimate the expression yield.

6. Determine the optimal [Mg^{2+}] and [K^+] concentrations, i.e., those that result in the highest expression level of the target protein.

PREPARATION OF *E. COLI* S12 EXTRACT

Cell-free expression systems have been reported based on crude cell extracts of diverse origin, such as *E. coli* cells (Sun et al., 2013), wheat germ embryos (Lee and Lassalle, 2011), rabbit reticulocytes (Anastasina et al., 2014), yeast (Wu and Sachs, 2014), insect cells (Stech et al., 2014), and human HeLa cells (Kobayashi et al., 2014). However, *E. coli*-derived cell extracts are still the most popular system due to the easy availability and standardized protocols. In this support protocol, we describe the preparation of *E. coli* S12 cell lysates that are suitable for the setup of in vitro coupled transcription and translation reactions.

Since the development of the first cell-free system for protein synthesis by Zamecnik in 1958 (Hoagland et al., 1958), different protocols for the preparation of *E. coli* cell extracts have been used and optimized. The protocol established by Pratt (1984) is used as the standard method of preparing the cell extract (generally referred to as the S30 extract) from *E. coli* cells.

The normal preparation of the S30 extract consists of many stages that are time-consuming, labor-intensive, or expensive. These steps include, for instance, cell fermentation, cell harvesting and washing, cell lysis by high-pressure homogenization, clarification of the lysate by high-speed centrifugation, a run-off step to minimize background protein expression, and several dialysis steps. The protocol presented here is a simplified and optimized method to produce the *E. coli* cell lysate (S12 extract). The protocol is based on the protocol described by Kim et al. (2006), with some minor modifications.

The *E. coli* BL21(DE3)Star strain, which is used for extract preparation, contains loss-of-function mutations in OmpT endoproteinase and lon protease genes. This results in greater stability for translated proteins that would otherwise be degraded by proteases if expressed in vivo. In addition, this strain carries a mutated rne gene (rne131) which encodes a truncated RNase E enzyme that lacks the ability to degrade mRNA, resulting in increased mRNA stability.

Materials

Superior Broth (Athena Enzyme Systems)
E. coli strain BL21 Star (DE3) glycerol stock (Life Technologies)
S12 extract buffer A (see recipe)
S12 extract buffer B (see recipe)

Ultra Yield Flask, 2.5 liter (Thomson)
Autoclave indicator tape
Ultra Yield Flask Air-Porous Seal (Thomson)
Temperature-controlled shaking incubator
Spectrophotometer (capable of measuring absorbance at 600 nm)
Refrigerated centrifuge
French pressure cell or high-pressure cell homogenizer

Preparation of Superior Broth medium

1. Prepare 10 liters of Superior Broth according to the manufacturer's instructions.
2. Pour the medium into Thomson Ultra Yield 2.5-liter flasks (1 liter culture medium/flask). Wrap mouth of flask with aluminum foil and place a small piece of autoclave indicator tape on top.
3. Autoclave the medium and store at room temperature.

Because the growth rate of the culture determines the ribosomal content of the extract (Zawada and Swartz, 2006), a medium that supports rapid cell growth should be used for best results. In our hands, Superior Broth medium produces the most efficient extracts, but other rich media such as TB or 2YT can be used as well.

Cell growth and harvest

4. Inoculate 250 ml sterile Superior Broth with 250 µl of an *E. coli* BL21 Star (DE3) glycerol stock and incubate overnight (37°C, 250 rpm). Be careful about contamination, since no antibiotic selection marker is added.

   For the preparation of the S12 extract, various other E. coli strains like MRE600, A19, or D10 can be used. A strain with reduced RNase activity (i.e., rna and/or rne mutations) may yield better results.

   IMPORTANT NOTE: *Do not use pLysS!*

5. The next morning, transfer 20 ml of the overnight grown *E. coli* preculture into each of the ten baffled Thomson Ultra Yield 2.5-liter flasks containing 1 liter sterilized Superior Broth medium. Seal the flasks with air-porous seals and incubate (37°C, 250 rpm).

6. Monitor cell growth by measuring A_{600}.

7. Grow the cells until they reach mid-logarithmic phase ($A_{600} = 4$ to 4.5).

   It is important to harvest the cells in mid-logarithmic phase because rapidly growing cells contain more active ribosomes, and extracts are therefore more productive. It is therefore recommended to perform an initial growth curve experiment to record the growth kinetics.

8. Chill the cell cultures as quickly as possible by placing them into a sink or tub filled with ice water.

9. Cool the cultures to below 12°C, then harvest the cells by centrifugation for 15 min at 7000 × *g*, 4°C.

   Cells should always be kept on ice from this point on.

10. Weigh the cell pellet.

11. Wash the cells by resuspending the cell pellet in 20 ml of S12 extract buffer A per gram of wet cell weight. Resuspend the cells by gently shaking or magnetic stirring in the cold room. Do not pipet the cells up and down to resuspend them, as this may result in unwanted cell breaking.

12. Centrifuge the resuspended cells 15 min at 7000 × *g*, 4°C.

13. Discard supernatant and repeat steps 11 and 12 two more times.

14. Weigh the cell pellet and store at −80°C.

Preparation of the S12 cell extract

15. Thaw the cell pellet on ice (or at 4°C) and resuspend the cells in 1.27 ml of freshly prepared, pre-chilled S12 extract buffer B per gram wet cell weight.

16. Lyse the cells by a single passage through a French pressure cell or a high pressure homogenizer at a constant pressure of 20,000 psi. Keep the lysate on ice during and after cell lysis.

17. Centrifuge the cell lysate 10 min at 12,000 × *g*, 4°C.

18. Transfer the supernatant to a clean tube and incubate at 37°C for 30 min.

This short incubation step reduces the background expression and improves the translational activity of the centrifuged lysate.

19. Aliquot the S12 cell extract on ice (e.g., 100, 250, and 500 µl fractions in microcentrifuge tubes) and store at −80°C.

    Because repeated freeze-thaw cycles decrease the translational activity of the cell extract, single-use aliquots should be prepared so that, once thawed, the extract will not have to be refrozen.

PREPARATION OF *E. COLI* S12 T7 RNA POLYMERASE EXTRACT

SUPPORT PROTOCOL 2

Efficient transcription in *E. coli* cell-free expression systems is usually accomplished by the addition of bacteriophage T7 RNA polymerase to the reaction. Highly purified T7 RNA polymerase is commercially available from many suppliers, but beside the high cost, the concentration of these stock solutions is often too low to obtain the required final polymerase concentration in the reaction. Instead, T7 RNA polymerase can be overproduced in *E. coli* and purified using a simple, one-step protocol (Rio, 2013). However, it is more convenient to use an *E. coli* lysate with endogenously overexpressed T7 RNA polymerase (S12-T7 extract), since this eliminates the need to add purified T7 RNA polymerase to the cell-free reaction. It should be noted that S12-T7 extracts are generally less productive than the standard S12 extract; therefore, it is recommended to use a combination of both lysates in the cell-free reaction.

The method for the preparation of the *E. coli* S12-T7 extract is similar to Support Protocol 1, but contains an additional IPTG induction step at step 6.

Additional Materials *(also see Support Protocol 1)*

Isopropyl-β-D-thiogalactopyranoside (IPTG)

Perform steps 1 to 5 of Support Protocol 1, then perform the following variations on step 6 and continue with step 7:

6a. After the inoculation of 10 baffled Thomson Ultra Yield 2.5 liter flasks containing 1 liter sterilized Superior Broth medium, monitor cell growth by measuring A_{600}.

6b. When the cell density (A_{600}) reaches 0.6, add IPTG to a final concentration of 1 mM to induce T7 RNA polymerase expression.

DESIGN AND PREPARATION OF DNA TEMPLATES FOR CELL-FREE MEMBRANE PROTEIN EXPRESSION

BASIC PROTOCOL 2

Since T7 RNA polymerase is used for transcription in most *E. coli* cell-free systems, any template encoding a DNA sequence under control of the T7 promoter can be used. Other key elements of the DNA template are Epsilon enhancer of T7 phage gene g10, a ribosome-binding site (RBS), the coding region of the target protein, and a T7-terminator region (Fig. 29.14.2).

DNA templates can be provided either as plasmids or as linear templates, such as polymerase chain reaction (PCR) fragments. The use of linear PCR products as template is attractive for screening and high-throughput applications, and because time-consuming cloning steps can be avoided. For preparative-scale expression, however, circular plasmid DNA templates are recommended because they are more stable than linear DNA templates. Frequently used T7-promoter-driven vectors for cell-free expression include pET (Novagen), pDEST (Life Technologies), or pIVEX (Roche Diagnostics) derivatives. Furthermore, these vector systems offer the option to fuse the target protein with a variety of terminal tags that might increase expression levels or facilitate purification. For difficult-to-express targets, we routinely use a tag variation screen by fusing expression

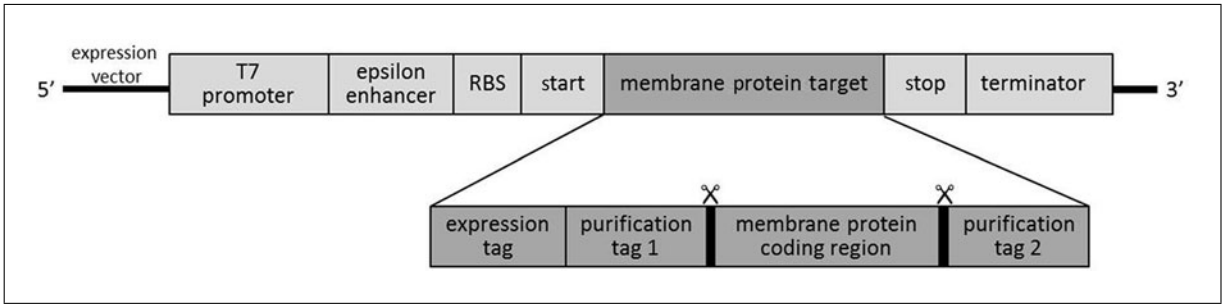

Figure 29.14.2 DNA template generation for protein expression in *E. coli*–based cell-free expression systems. Essential T7 regulatory sequences are shaded in light gray; translated elements are highlighted in dark gray. Protease cleavage sites are indicated by a scissor symbol.

tags to the N-terminus of the target protein in combination with one or more cleavable N- or C-terminal purification tags (see Fig. 29.14.2 and Table 29.14.3). The expression tags are optimized in suppressing secondary structure formation, which may prevent the initiation of translation and/or have been reported to improve the cell-free expression of membrane proteins (Ahn et al., 2007; Haberstock et al., 2012; Lyukmanova et al., 2012; Zhang et al., 2014).

In addition to the sequence elements surrounding the start codon, variations in codon usage may also affect the level of protein expression. If the gene of interest contains codons that are rarely used in *E. coli*, its expression may result in the depletion of rare codon tRNAs and premature termination events, eventually leading to poor expression levels. Especially when clusters of rare codons are present in the mRNA transcript, codon bias may become highly problematic and even result in undetectable expression levels (Kane, 1995).

If the overall rare codon content is suspected to result in poor or incomplete expression of the target gene, expression levels can be optimized by lowering the expression temperature (e.g., to 16° to 20°C) or by supplementing the cell-free reaction mixture with the corresponding minor tRNAs (Chumpolkulwong et al., 2006). The use of a codon-optimized synthetic gene is another attractive strategy that can be considered to improve the expression yield.

Amplification and purification of the DNA template

High-purity plasmid DNA is the key to successful transcription and subsequent translation in *E. coli* cell-free systems. We therefore recommend using purified plasmids obtained from commercially available DNA preparation kits, which are usually sufficiently pure (A_{260}/A_{280} absorption ratio > 1.7) to be used as DNA template in cell-free reactions. Template stock solutions should be prepared with RNase-free water and concentrations should be between 200 and 500 ng/μl. For optimal expression efficiency, the final template concentration should be determined for each new protein target with an initial concentration screen over a range of 0.1 to 20 ng DNA/μl.

Materials

Cell-free expression plasmid (e.g., pIVEX2.4 d)
DNA encoding the gene of interest (e.g., a genomic or cDNA library)

Additional reagents and equipment for the polymerase chain reaction (PCR; Kramer and Coen, 2001), DNA sequencing (Shendure et al., 2011), and general molecular cloning techniques (Ausubel et al., 2015)

Table 29.14.3 Expression and Purification Fusion Tags for the Cell-Free Expression of Membrane Proteins

Tag name	Nucleotide sequence	Amino acid sequence	Residues	Mol. wt. (Da)
Expression tags				
AT4	ATGAAATATTATAAA	MKYYK	5	731.9
G	ATGAAAAGTAAAGGAGAAGAA	MKSKGEE	7	807.9
H	ATGAAACCATACGATGGTCCA	MKPYDGP	7	806.9
HpTrxA	ATGGGATCTGATAAAATTATTCATCTGACTGAT GATTCTTTTGATACTGATGTACTTAAGGCAGAT GGTGCAATCCTGGTTGATTTCTGGGCACACTGG TGCGGTCCGTGCAAAATGATCGCTCCGATTCTG GATGAAATCGCTGACGAATATCAGGGCAAACTG ACCGTTGCAAAACTGAACATCGATCACAACCCG GGCACTGCGCCGAAATATGGCATCCGTGGTATC CCGACTCTGCTGCTGTTCAAAAACGGTGAAGTG GCGGCAACCAAAGTGGGTGCACTGTCTAAAGGT CAGTTGAAAGAGTTCCTCGACGCTAACCTGGCC	MGSDKIIHLTDDSFDTD VLKADGAILVDFWAHWC GPCKMIAPILDEIADEY QGKLTVAKLNIDHNPGT APKYGIRGIPTLLLFKN GEVAATKVGALSKGQLK EFLDANLA	111	11880.7
Mistic	ATGTTTTGTACATTTTTTGAAAAACATCACCGG AAGTGGGACATACTGTTAGAAAAAAGCACGGGT GTGATGGAAGCTATGAAAGTGACGAGTGAGGAA AAGGAACAGCTGAGCACAGCAATCGACCGAATG AATGAAGGACTGGACGCGTTTATCCAGCTGTAT AATGAATCGGAAATTGATGAACCGCTTATTCAG CTTGATGATGATACAGCCGAGTTAATGAAGCAG GCCCGAGATATGTACGGCCAGGAAAAGCTAAAT GAGAAATTAAATACAATTATTAAACAGATTTTA TCCATCTCAGTATCTGAAGAAGGAGAAAAAGAA	MFCTFFEKHHRKWDILL EKSTGVMEAMKVTSEEK EQLSTAIDRMNEGLDAF IQLYNESEIDEPLIQLD DDTAELMKQARDMYGQE KLNEKLNTIIKQILSIS VSEEGEKE	111	12832.5
T7	ATGACCCATTTGCTGTCCACCCGTCATGCTAGC CAT	MTHLLSTRHASH	12	1390.6
pelB	ATGAAATACCTATTGCCTACGGCAGCCGCTGGA TTGTTATTACTCGCTGCCCAACCAGCGATGGCC	MKYLLPTAAAGLLLLAA QPAMA	22	2228.8
ompA	ATGAAAAAGACAGCTATCGCGATTGCAGTGGCA CTGGCTGGTTTCGCTACCGTAGCGCAGGCC	MKKTAIAIAVALAGFAT VAQA	21	2046.5
phoA	ATGAAACAAAGCACTATTGCACTGGCACTCTTA CCGTTACTGTTTACCCCTGTGACAAAAGCC	MKQSTIALALLPLLFTP VTKA	21	2256.8
malE	ATGAAAATAAAAACAGGT GCACGCATCCTCGCA TTATCCGCATTAACGACGATGATGTTTTCCGCC TCGGCTCTCGCC	MKIKTGARILALSALTT MMFSASALA	26	2698.3
ompC	ATGAAAGTTAAAGTACTGTCCCTCCTGGTCCCA GCTCTGCTGGTAGCAGGCGCAGCAAACGCT	MKVKVLSLLVPALLVAG AANA	21	2078.6
ompT	ATGCGGGCGAAACTTCTGGGAATAGTCCTGACA ACCCCTATTGCGATCAGCTCTTTTGCT	MRAKLLGIVLTTPIAIS SFA	21	2102.6

continued

Table 29.14.3 Expression and Purification Fusion Tags for the Cell-Free Expression of Membrane Proteins, *continued*

Tag name	Nucleotide sequence	Amino acid sequence	Residues	Mol. wt. (Da)
heposp	ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGG CTTCTCCTGTCCCTGCTGTCGCTCCCTCTGGGC CTCCCAGTCCTGGGC	MGVHECPAWLWLLLSLL SLPLGLPVLG	27	2928.6
S	ATGAAAGAAACCGCTGCTGCTAAATTCGAACGC CAGCACATGGACAGC	MKETAAAKFERQHMDS	16	1880.1
ESR	ATGGAAGAGGTCAACCTG	MEEVNL	6	733.8
Detection/purification tags				
SUMO	ATGTCGGACTCAGAAGTCAATCAAGAAGCTAAG CCAGAGGTCAAGCCAGAAGTCAAGCCTGAGACT CACATCAATTTAAAGGTGTCCGATGGATCTTCA GAGATCTTCTTCAAGATCAAAAAGACCACTCCT TTAAGAAGGCTGATGGAAGCGTTCGCTAAAAGA CAGGGTAAGGAAATGGACTCCTTAAGATTCTTG TACGACGGTATTAGAATTCAAGCTGATCAGACC CCTGAAGATTT GGACATGGAGGATAACGATATT ATTGAGGCTCACAGAGAACAGATTGGTGGT	MSDSEVNQEAKPEVKPE VKPETHINLKVSDGSSE IFFKIKKTTPLRRLMEA FAKRQGKEMDSLRFLYD GIRIQADQTPEDLDMED NDIIEAHREQIGG	98	11261.7
LUMIO	TGTTGTCCTGGCTGTTGC	CCPGCC	6	584.7
His10	CACCACCACCACCACCACCACCACCAC	HHHHHHHHHH	11	1389.4
FLAG	GACTACAAGGACGACGATGACAAG	DYKDDDDK	8	1013.0
Rho1D4	ACCGAGACTTCCCAGGTGGCGCCAGCT	TETSQVAPA	9	903.0

DNA template generation

For efficient cell-free expression, all DNA templates must contain a T7 promoter, an initiation codon, and a prokaryotic Shine-Dalgarno sequence upstream of the gene of interest. Although linear DNA fragments (e.g., PCR products) can be used as template DNA in the cell-free reaction, supercoiled plasmid DNA is recommended to obtain the highest expression yields.

1. Design and synthesize PCR primers (see Kramer and Coen, 2001) to amplify the gene of interest from a DNA template (e.g., genomic DNA or cDNA) and to simultaneously introduce the desired N- or C-terminal expression and/or purification tags and appropriate restriction sites for direct subcloning into an expression vector that is compatible with *E. coli* cell-free expression systems (e.g., pET or pIVEX vector).
2. Amplify the cDNA sequences by PCR (Kramer and Coen, 2001) and clone the purified DNA inserts into the cell-free expression vector using standard molecular cloning techniques
3. Perform DNA sequence analysis (Shendure et al., 2011) to confirm the correct assembly and sequence of the generated expression construct(s).

An alternative approach involves the design and construction of a synthetic gene that encodes the target membrane protein fused to the desired expression and/or purification tags. Such a strategy offers the possibility to optimize certain features of the gene that might influence its expression, such as codon usage or GC content.

CELL-FREE EXPRESSION OF MEMBRANE PROTEINS AS PRECIPITATES IN BATCH CONFIGURATION

The open nature of cell-free protein expression systems offers the opportunity to overexpress membrane proteins in different hydrophobic environments, like detergent micelles, liposomes, or nanodiscs (Bernhard and Tozawa, 2013). A simpler and faster approach is the cell-free production of membrane proteins in the absence of membrane mimetics, which results in the instant precipitation of the synthesized membrane protein in the reaction mix (precipitate-based cell-free expression; P-CF). Although precipitated membrane proteins produced in P-CF mode might resemble inclusion body formation in conventional cellular expression systems, these precipitates can usually be solubilized efficiently within a few hours by the addition of relatively mild detergents, without applying classical refolding procedures.

Because the expression level of membrane proteins is often reduced in the presence of detergents (D-CF) or lipids (L-CF), the P-CF mode is recommended as the first choice for the initial screening of new membrane protein targets, for protocol development, and for product yield optimization (Haberstock et al., 2012). Furthermore, the P-CF setup has also been proven valuable for the production of membrane proteins for NMR analysis (Maslennikov et al., 2010; Sobhanifar et al., 2010; Klammt et al., 2012), for drug screening (Lindert et al., 2014), and for the screening of membrane protein libraries (Langlais et al., 2007; Schwarz et al., 2010). In this basic protocol, we describe a procedure for the cell-free expression of membrane proteins as precipitates in batch configuration (Fig. 29.14.3). In this configuration, reactions are performed in a single compartment containing all necessary components for cell-free protein synthesis. As a consequence, batch reactions are easy to handle and enable a fast and reliable synthesis of a given target protein, making it a particular useful approach for high-throughput applications, as the reaction can be carried out in microtiter plates with reaction volumes of just a few microliters. The disadvantage of this configuration is the reaction time, which is limited to few hours due to the rapid depletion of energy resources and the accumulation of inhibitory byproducts such as free phosphates, resulting in limited protein yields.

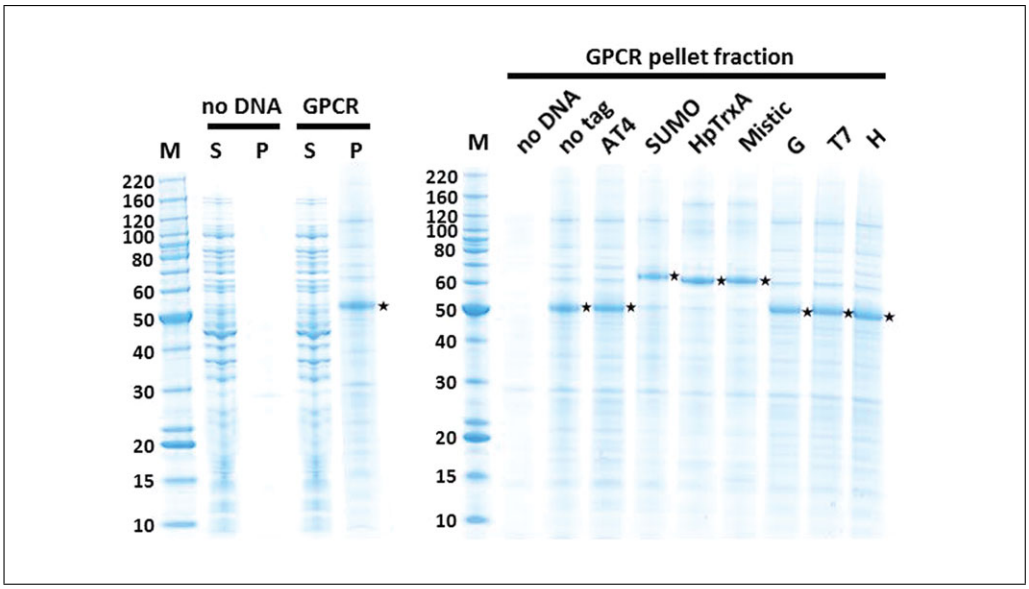

Figure 29.14.3 Cell-free expression of different GPCR constructs in batch P-CF mode. Cell-free reactions were performed in analytical-scale reactions in batch P-CF mode, as described in Basic Protocol 3. Soluble (S; 1.5 μl loaded) and resuspended pellet fractions (P; 5 μl loaded) were separated by SDS-PAGE and stained with Coomassie blue. The recombinant GPCR fusion proteins are indicated with an asterisk. The marker (M) is given in kilodaltons.

Table 29.14.4 Reaction Mix Preparation for the Cell-Free Expression of Membrane Proteins in Batch P-CF Mode (70-μl reactions)

Component (unit)	Stock concentration	Final concentration	Volume (μl)
NaN_3 (mg/ml)	100	0.5	0.35
PEG8000 (mg/ml)	400	20	3.50
K^+ acetate; KOAc (mM)	4000	40	0.70
HEPES (M)	2.4	0.1	2.92
Complete protease inhibitor cocktail without EDTA (×)	50	1	1.40
Folinic acid calcium salt (mg/ml)	10	0.1	0.70
NTP-mix (×)	27.78	1	2.52
Phospo(enol)pyruvic acid monopotassium salt; PEP (mM)	430	20	3.26
Acetyl phosphate lithium potassium salt; AcP (mM)	1000	20	1.40
Amino acid mix (mM)	4	0.5	8.75
RCWMDE-mix (mM)	16.7	1	4.19
Magnesium acetate tetrahydrate; $Mg(OAc)_2$ (mM)	1000	5.1	0.36
Pyruvate kinase (mg/ml)	10	0.04	0.28
Membrane protein template DNA (ng/μl)	200	15	5.25
Ribolock (U/μl)	40	0.3	0.53
tRNA *E. coli* (mg/ml)	40	0.5	0.88
S12 extract (%)	100	17.5	12.25
S12-T7 extract (%)	100	17.5	12.25
Milli-Q water (RNase free)			8.53
Total reaction mix volume (μl)			70.0

Using an optimized system however, yields between 400 and 600 μg/ml can be obtained routinely.

Materials

E. coli S12 cell extract (see Support Protocol 1)
E. coli S12-T7 cell extract (see Support Protocol 2)
100 mg/ml NaN_3 (Sigma)
400 mg/ml PEG8000 (Sigma)
4 M potassium acetate (KOAc; Sigma)
2.4 M HEPES (Sigma; adjust to pH 8.0 with KOH)
50× Complete protease inhibitor cocktail without EDTA (Roche Diagnostics); prepare by dissolving ten tablets in 10 ml of Milli-Q water
10 mg/ml folinic acid calcium salt (Sigma)
NTP mix (33.3 mM ATP, 22.2 mM UTP, 22.2 mM GTP, 22.2 mM CTP; see recipe)
430 mM phospo(enol)pyruvic acid monopotassium salt (PEP; Sigma); adjust to pH 7.0 with KOH)
1 M acetyl phosphate lithium potassium salt (AcP; Sigma; adjust to pH 7.0 with KOH)

Amino acid mix (4 mM each; see recipe)
RCWMDE amino acid mix (16.7 mM each, see recipe)
10 M magnesium acetate tetrahydrate ($Mg(OAc)_2$; Sigma)
10 mg/ml pyruvate kinase (Roche Diagnostics)
40/μl Ribolock RNase (ribonuclease) inhibitor (Thermo Scientific)
40 mg/ml *E. coli* total transfer RNA (tRNA; Roche Diagnostics)
2.8 M potassium acetate (KOAc; anhydrous; Sigma)
200 ng/μl plasmid DNA encoding the membrane protein target
Solubilization buffer: 20 mM Tris·Cl, pH 7.5/150 mM NaCl containing 1× Complete protease inhibitor cocktail without EDTA (Roche Diagnostics)

Rolling device or temperature-controlled shaking incubator
Refrigerated microcentrifuge

Additional reagents and equipment for SDS-PAGE (Gallagher, 2012) and western blotting (immunoblotting; Gallagher et al., 2012)

1. Thaw an aliquot of the S12 and S12-T7 extracts and all the cell-free reaction components listed in Table 29.14.4.

   Enzymes and extracts should be thawed on ice.

2. Use the pipetting scheme in Table 29.14.4 to prepare the cell-free reaction(s).

   The amounts given are for a standard 70-μl reaction; if you are scaling up the reaction, adjust the volume of reagents accordingly.

3. Incubate the reactions for 3 to 5 hr with gentle shaking or rolling at 30°C.

4. Harvest the precipitated membrane protein by microcentrifuging for 10 min at 18,000 × *g*, 4°C. Carefully remove the supernatant, taking care not to disturb the protein pellet at the bottom.

5. Resuspend the protein pellet with solubilization buffer in a volume similar to that of the initial cell-free reaction.

6. Analyze supernatant and pellet fractions separately by SDS-PAGE (Gallagher, 2012) and western blotting (Gallagher et al., 2008), if necessary. For SDS-PAGE analysis, load 1 to 2 μl of the supernatant and 5 to 10 μl of the resuspended protein pellet. For western blot analysis, use similar volumes of a 1:40 dilution of the supernatant and resuspended pellet fraction.

CELL-FREE EXPRESSION OF MEMBRANE PROTEINS AS PRECIPITATES IN CONTINUOUS-EXCHANGE CONFIGURATION

BASIC PROTOCOL 4

Although the batch mode setup is very useful for analytical purposes, such as the screening of DNA constructs or preliminary feasibility studies, it is not the method of choice when large amounts of protein are required. The rapid depletion of substrates and the accumulation of inhibitory waste products in the reaction mix shorten the life time of the system, which limits the final protein yield.

A more convenient setup for preparative-scale protein production is the continuous-exchange cell-free (CECF) configuration. The CECF mode requires two compartments that are separated by a semi-permeable dialysis membrane. One of these compartments holds the cell-free reaction mix that contains all the essential components for the transcription/translation process. The second chamber contains a feeding mix that holds all lower-molecular-weight components, and supplies the reaction mix with ions, energy substrates, nucleotides, and amino acids. In turn, the inhibitory by-products formed in

the reaction mix are efficiently diluted via the membrane into the feeding mix. This results in extended reaction times (usually 20 to 24 hr) and higher levels of produced protein (typically in the mg/ml range).

Materials

E. coli S12 cell extract (see Support Protocol 1)
E. coli S12-T7 cell extract (see Support Protocol 2)
100 mg/ml NaN_3 (Sigma)
400 mg/ml PEG8000 (Sigma)
4 M potassium acetate (KOAc; Sigma)
2.4 M HEPES (Sigma; adjust to pH 8.0 with KOH)
50× Complete protease inhibitor cocktail without EDTA (Roche Diagnostics); prepare by dissolving ten tablets in 10 ml of Milli-Q water
10 mg/ml folinic acid calcium salt (Sigma)
NTP mix (33.3 mM ATP, 22.2 mM UTP, 22.2 mM GTP, 22.2 mM CTP; see recipe)
430 mM phospo(enol)pyruvic acid monopotassium salt (PEP; Sigma; adjust to pH 7.0 with KOH)
1 M acetyl phosphate lithium potassium salt (AcP; Sigma; adjust to pH 7.0 with KOH)
Amino acid mix (4 mM each; see recipe)
RCWMDE amino acid mix (16.7 mM each, see recipe)
1 M magnesium acetate tetrahydrate [$Mg(OAc)_2$; Sigma]
10 mg/ml pyruvate kinase (Roche Diagnostics)
40/µl Ribolock RNase (ribonuclease) inhibitor (Thermo Scientific)
40 mg/ml *E. coli* total transfer RNA (tRNA; Roche Diagnostics)
2.8 M potassium acetate (KOAc; anhydrous; Sigma)
200 ng/µl plasmid DNA encoding the membrane protein target
Solubilization buffer: 20 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)/150 mM NaCl containing 1× Complete protease inhibitor cocktail without EDTA (Roche Diagnostics)

Plastic vials, Ø 15 mm (GE Healthcare, BR-1006-54)
D-Tube Dialyzer Mini (MWCO 12 to 14 kDa; Novagen)
Rubber caps, type 5 (GE Healthcare, BR-1006-55)
Rolling device or temperature-controlled shaking incubator

Additional reagents and equipment for SDS-PAGE (Gallagher, 2012) and western blotting (immunoblotting; Gallagher et al., 2012)

Setting up the CECF reaction

Cell-free expression in continuous-exchange mode requires the preparation of a reaction mix (RM) and a feeding mix (FM). The RM:FM volume ratio is an important parameter for the final yield of recombinant protein, and is usually between 1:10 and 1:30. Higher feeding-mix volumes will certainly increase product yields, but not in a linear correlation. Considering the relatively high costs of the precursors in the feeding mix, RM:FM ratios between 1:14 and 1:17 are recommended to provide good yields at moderate cost.

1. Thaw all stock solutions and mix carefully. Keep all solutions on ice after thawing.
2. First, prepare a master mix of all components that are common to the feeding and reaction mix (see Table 29.14.5; "FRM master mix"). Gently mix the solution by inverting the tube several times.

Table 29.14.5 Preparation of the Reaction Mix (230 μl) and Feeding Mix (4 ml) for the Cell-Free Expression of Membrane Proteins in P-CECF Mode

Component (unit)	Stock concentration	Final concentration	Volume (μl)
FRM master mix			
NaN_3 (mg/ml)	100	0.5	23.6
PEG8000 (mg/ml)	400	20	236.4
K^+ acetate; KOAc (mM)	4000	80	94.6
HEPES (M)	2.4	0.1	197.0
Complete protease inhibitor cocktail without EDTA (×)	50	1	94.6
Folinic acid calcium salt (mg/ml)	10	0.1	47.3
NTP-mix (fold)	27.78	1	170.2
Phospo(enol)pyruvic acid monopotassium salt; PEP (mM)	430	20	219.9
Acetyl phosphate lithium potassium salt; AcP (mM)	1000	20	94.6
RCWMDE-mix (mM)	16.7	1	283.1
Magnesium acetate tetrahydrate; $Mg(OAc)_2$ (mM)	1000	7.1	33.6
Total FRM master mix volume (μl)			1494.8
Feeding mix			
Master mix FRM			1265.9
S12 extract buffer B (without DTT) (%)	100	35	1400
Amino acid mix (mM)	4	1	1000
Milli-Q water			334.1
Total feeding mix volume (μl)			4000.0
Reaction mix			
Master mix FRM			72.63
Amino acid mix (mM)	4	0.5	28.75
Pyruvate kinase (mg/ml)	10	0.04	0.92
Membrane protein template DNA	200	15	17.25
Ribolock (U/μl)	40	0.3	1.725
tRNA *E. coli* (mg/ml)	40	0.5	2.875
S12 extract (%)	100	17.5	40.25
S12-T7 extract (%)	100	17.5	40.25
Milli-Q water			25.35
Total reaction mix volume (μl)			230

3. Transfer the required FM volume from the FRM master mix to a separate tube. Complete the FM with S12 extract buffer B without DTT, the amino acid mixture and Milli-Q water (see Table 29.14.5; "Feeding Mix"). Pre-incubate the FM at 30°C.
4. Prepare the RM in a microcentrifuge tube on ice. Carefully mix all components; do not vortex (see Table 29.14.5; "Reaction mix").

5. Fill the FM container (plastic vial, Ø 15 mm) with the FM.
6. Fill a D-Tube Dialyzer Mini (MWCO 12 to 14 kDa) with the RM. Try to avoid air bubbles, which might interfere with an efficient exchange between the RM and FM compartments. Screw on the cap and seal with Parafilm.
7. Place the filled D-tube Dialyzer into the vial that contains the FM. Close the FM container with the rubber cap and seal with Parafilm.
8. Incubate the reaction on a rolling device or an appropriate shaker with moderate agitation at 30°C for 15 to 20 hr.
9. Harvest the membrane protein by microcentrifugation for10 min at 18,000 × *g*, 4°C. Carefully remove the supernatant, taking care not to disturb the pellet at the bottom.
10. Resuspend the protein pellet with solubilization buffer in a volume similar to that of the initial cell-free reaction mix.
11. Analyze supernatant and pellet fractions separately by SDS-PAGE (Gallagher, 2012) and western blotting (Gallagher et al., 2008), if necessary. For SDS-PAGE analysis, load 1 to 2 µl of the supernatant and 5 to 10 µl of the resuspended protein pellet. For western blot analysis, use similar volumes of a 1:40 dilution of the supernatant and pellet fraction.

BASIC PROTOCOL 5

CELL-FREE PRODUCTION OF SOLUBLE MEMBRANE PROTEINS

Different expression strategies for the cell-free production of soluble membrane proteins are possible. A first strategy encompasses the solubilization of precipitated membrane proteins that were synthesized in P-CF mode. In contrast to proteins present in inclusion bodies, cell-free-produced precipitates of membrane proteins do not seem to be completely unfolded. As a consequence, functionally active membrane proteins can be obtained by resolubilization of the protein precipitates in relatively mild detergents.

Another strategy is to add a membrane mimetic (e.g., detergents, liposomes or nanodiscs) to the reaction mix and to synthesize the membrane proteins directly into a well-defined hydrophobic environment. In this basic protocol, we will describe a method for the solubilization of P-CF expressed membrane proteins (Fig. 29.14.4). In addition, we will provide protocols for the cell-free production of membrane proteins in the presence of detergents or amphipathic polymers (Alternate Protocol 1; Fig. 29.14.5), and we will briefly discuss cell-free protein synthesis in the presence of lipids (Alternate Protocol 2).

Materials

P-CF expressed membrane protein (see Basic Protocols 3 and 4) in solubilization buffer

Resuspension buffer: 20 mM Tris·Cl, pH 7.5 (*APPENDIX 2E*)/150 mM NaCl containing 1× Complete protease inhibitor cocktail without EDTA and 1 mM DTT

Solubilization buffer: same as resuspension buffer, but supplemented with an appropriate detergent (high-purity grade); detergent concentration should be higher than the specific critical micellar concentration (CMC), e.g., 3- to 5-fold higher than their respective CMC.

Refrigerated microcentrifuge

Rolling device or shaking incubator

Additional reagents and equipment for SDS-PAGE (Gallagher, 2012) and western blotting (immunoblotting; Gallagher et al., 2012)

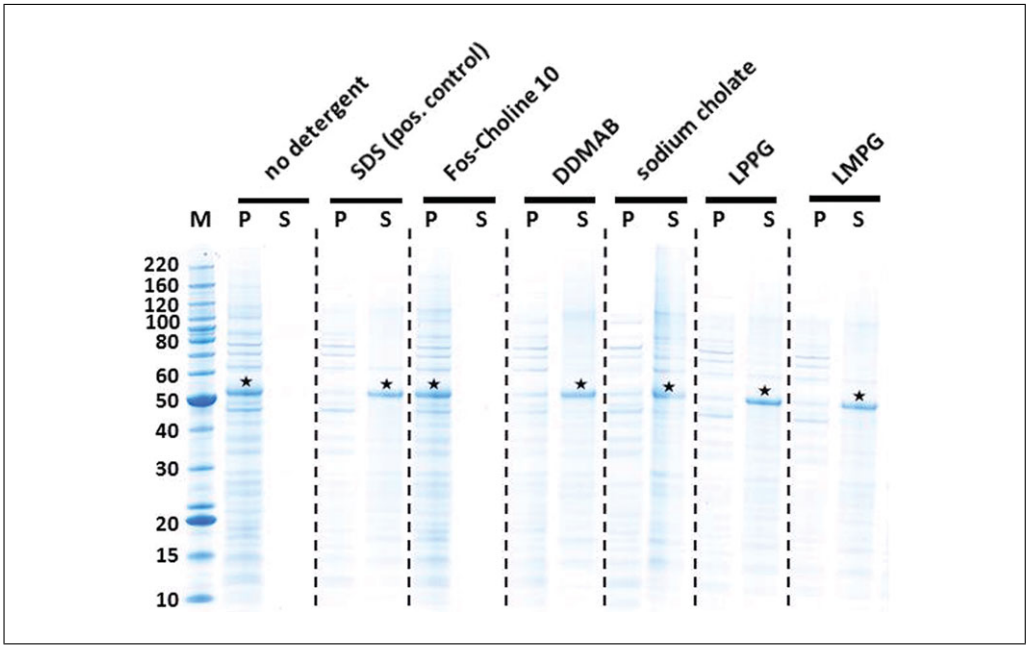

Figure 29.14.4 Solubilization screen of a P-CF expressed GPCR. Protein precipitates resulting from 70-μl cell-free batch reactions were resuspended in 70 μl solubilization buffer containing 2% (w/v) of the following detergents (high-purity grade): sodium *n*-dodecyl sulfate (SDS); *n*-decylphosphocholine (Fos-Choline 10); (N-dodecyl-N,N-dimethylammonio)butyrate (DDMAB) ; sodium cholate; 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LPPG); and 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LMPG). Non-solubilized protein was precipitated by centrifugation and supernatant (S) and resuspended pellet fractions (P) were separated by SDS-PAGE, followed by Coomassie blue staining. Asterisks indicate the expressed GPCR.

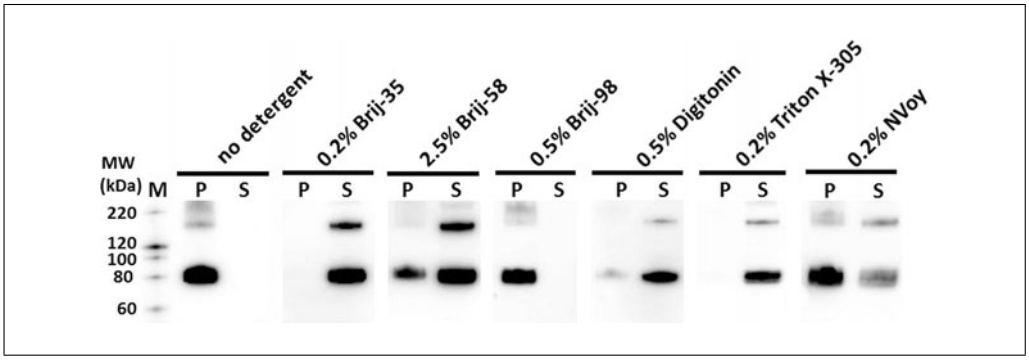

Figure 29.14.5 Cell-free expression of a GPCR in the presence of detergents. Proteins were synthesized in the presence of the indicated concentrations of detergents or the NVoy polymer, as described in Alternate Protocol 1. Soluble (S) and resuspended pellet fractions (P) were separated by SDS-PAGE (10 μl of a 1:40 dilution was loaded) and analyzed by western blotting with an anti-FLAG antibody. Primary and secondary antibodies were used at a 1:3000 dilution of a 1 mg/ml stock solution. Efficient soluble expression was observed in the presence of 0.2% Brij-35 (w/v), 0.5% digitonin (w/v), and 0.2% (w/v) Triton X-305, whereas the detergents Brij-58, Brij-98, and the NVoy polymer do not seem to be suitable for the soluble expression of this protein.

1. Microcentrifuge the solubilization buffer suspension containing the desired membrane 10 min at 18,000 × *g*, 4°C.
2. To reduce co-precipitated *E. coli* proteins, discard the supernatant and wash the pellet in resuspension buffer by resuspending it in a volume similar to that of the initial reaction mix. Centrifuge 10 min at 18,000 × *g*, 4°C.
3. Discard the supernatant and repeat this washing step.

Table 29.14.6 Compounds Suitable for P-CF, S_D-CF and S_N-CF Expression of Membrane Proteins

Compound	Short name	Charge	Mol. wt. (Da)	CMC (%)	Final conc. (%)	Fold CMC	Expression mode
Detergents							
Sodium *n*-dodecyl sulfate	SDS	Ionic	288	0.24	2	8.4	P-CF
n-Dodecylphosphocholine (Fos-choline 12)	Fos-12	Zwitter-ionic	352	0.047	2	42.6	P-CF
n-Tetradecylphosphocholine (Fos-choline 14)	Fos-14	Zwitter-ionic	379	0.0046	2	434.8	P-CF
1-Palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)]	LPPG	Ionic	507	0.0009	2	261.5	P-CF
1-Myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)]	LMPG	Ionic	478	0.0076	2	2195.9	P-CF
N-Octyl-β-D-glucoside	β-OG	Non-ionic	292	0.52	2	3.8	P-CF
N-Dodecyl-N,N-(dimethylammonio)butyrate	DDMAB	Zwitter-ionic	300	0.129	2	15.5	P-CF
Lauryldimethylamine-oxide	LDAO	Zwitter-ionic	229	0.0458	2	43.7	P-CF
Polyoxyethylene-(23)-lauryl-ether	Brij-35	Non-ionic	1200	0.0096	0.5	52.1	S_D-CF
Polyoxyethylene-(20)-cetyl-ether	Brij-58	Non-ionic	1123	0.0084	1	118.7	S_D-CF
Polyoxyethylene-(20)-stearyl-ether	Brij-78	Non-ionic	1152	0.0053	1	188.7	S_D-CF
Polyoxyethylene-(20)-oleyl-ether	Brij-98	Non-ionic	1150	0.0029	0.2	69.6	S_D-CF
n-Dodecyl-β-D-maltoside	DDM	Non-ionic	511	0.0061	0.1	16.3	S_D-CF
n-Dodecyl-β-D-maltoside	DM	Non-ionic	483	0.0869	0.2	2.3	S_D-CF
PEG P-1,1,3,3-tetra-methyl-butylphenyl ether	TritonX-100	Non-ionic	650	0.0150	0.1	6.7	S_D-CF
Octylphenol ethoxylate	TritonX-305	Non-ionic	1526	0.0992	0.5	5.0	S_D-CF
Digitonin	Digitonin	Non-ionic	1229	0.0897	0.4	4.5	S_D-CF
Polymers							
NVoy polymer	NVoy	neutral	5000	N/A	0.25	N/A	S_N-CF

4. Resuspend the washed pellet in solubilization buffer with the appropriate detergent by pipetting up and down. Incubate for 2 hr at 30°C with shaking.
5. Centrifuge 10 min at 18,000 × g, 4°C, and resuspend the remaining precipitate in resuspension buffer (in the initial reaction mix volume).
6. Analyze the supernatant and the resuspended protein pellet by SDS-PAGE (Gallagher, 2012) and western blotting (Gallagher et al., 2008).

A detergent screen should be performed for each new membrane protein target to identify suitable detergents for solubilization. A list of commonly used detergents for the solubilization of P-CF-expressed membrane proteins is provided in Table 29.14.6.

ALTERNATE PROTOCOL 1

CELL-FREE EXPRESSION OF MEMBRANE PROTEINS IN THE PRESENCE OF DETERGENTS (S_D-CF) OR THE AMPHIPATIC POLYMER NVoy (S_N-CF)

If the solubilization of P-CF-synthesized membrane protein precipitates does not result in functionally folded proteins, co-translational solubilization of the expressed membrane proteins should be considered. The optimal detergent type and concentration have

to be determined experimentally for each particular membrane protein, taking into account that any detergent will become inhibitory to the cell-free system above a certain threshold concentration. Thus, the best compromise between solubilization efficiency and expression yield should be found.

A list of commonly used detergents for the soluble cell-free expression of membrane proteins and their working concentrations is given in Table 29.14.6. While some detergents such as *n*-decyl-β-D-maltoside (DM) already inhibit the cell-free reaction at concentrations close to their CMC, other detergents are tolerated up to 100-fold CMC without significant decrease in protein yield.

Although detergents provide a convenient way to solubilize membrane proteins, most detergents are destabilizing and can lead to denaturation and inactivation of the protein over time. Therefore, efforts have been made to design new solubilizing agents that are less destabilizing than traditional detergents, such as the NVoy polymer. NVoy is a nondetergent, polyfructose-based polymer that has been successfully employed for the soluble cell-free expression of various GPCRs from the three major subfamilies (Klammt et al., 2011). In this section, we describe a method for the soluble expression of membrane proteins in the presence of detergents (S_D-CF) or the NVoy polymer (S_N-CF) in batch and continuous-exchange mode.

Additional Materials *(also see Basic Protocols 3 and 4)*

Detergents: stock solutions of the selected detergents (high-purity grade) in Milli-Q water; the stock concentration should be at least 10 times higher than the final working concentration (see Table 29.14.6).
NVoy polymer (Expedeon)

S_D-CF and S_N-CF expression in analytical-scale reactions (batch mode)

1a. For initial trials, use the working concentrations indicated in Table 29.14.6. Prepare stock solutions of the selected detergents and the NVoy polymer in Milli-Q water.

Stock concentrations should be at least ten times higher than the final concentration indicated in Table 29.14.6.

Some detergents are difficult to dissolve in water, or may dissolve as an aggregate at room temperature. Such aggregates will precipitate out of solution when cooled to 4°C, resulting in imprecise detergent concentrations. It is therefore recommended to heat the detergent solution to 50°C and then cool back to room temperature. This should prevent re-precipitation at 4°C.

2a. Prepare a cell-free master mix as described for the P-CF expression in batch mode (see Basic Protocol 3), but add suitable amounts of detergent or NVoy to reach the desired final concentration.

The amount of added detergent/NVoy has to be subtracted from the Milli-Q volume in the pipetting scheme given in Table 29.14.4, and is thus limited by this volume.

3a. Incubate the reaction on an appropriate shaker at 30°C with moderate agitation for at least 3 hr.

4a. Microcentrifuge the cell-free reactions for 10 min at 18,000 × g, 4°C. Carefully remove the supernatant, taking care not to disturb the pellet at the bottom. Resuspend the protein pellet with resuspension buffer in a volume similar to that of the initial cell-free reaction.

5a. Analyze supernatant and pellet fractions separately by SDS-PAGE (Gallagher, 2012) and western blotting (Gallagher et al., 2008).

Successfully expressed S_D-CF or S_N-CF membrane proteins will preferentially stay in the supernatant after centrifugation of the cell-free reaction for 10 min at 18,000 × g. Since there are also many E. coli proteins in the soluble fraction, it depends on the expression level whether the target protein can be detected by SDS-PAGE followed by Coomassie blue staining. It is therefore recommended to verify protein expression by western blotting.

S_D-CF and S_N-CF expression in preparative-scale reactions (continuous-exchange mode)

1b. Prepare cell-free reactions as described for expression in continuous-exchange P-CF mode (see Basic Protocol 4) and add suitable amounts of detergent/NVoy to reach the desired final concentration.

The amount of added detergent/NVoy has to be subtracted from the Milli-Q volume in the pipetting scheme given in Table 29.14.5, and is thus limited by this volume. The detergent/NVoy should be added to the reaction mix and the feeding mix. Mix well by pipetting, but avoid air bubbles (especially in the reaction mix).

2b. Incubate the reaction on a rolling device or an appropriate shaker with moderate agitation at 30°C for 15 to 20 hr.

3b. Collect the reaction mix and centrifuge for 10 min at 18,000 × g, 4°C. Carefully remove the supernatant (which contains the solubilized membrane protein), taking care not to disturb the pellet at the bottom. Resuspend the protein pellet with resuspension buffer in a volume similar to that of the initial cell-free reaction.

4b. Analyze supernatant and pellet fractions separately by SDS-PAGE (Gallagher, 2012) and western blotting (Gallagher et al., 2008).

ALTERNATE PROTOCOL 2

CELL-FREE EXPRESSION OF MEMBRANE PROTEINS IN THE PRESENCE OF LIPIDS (L-CF)

Lipids represent the natural environment of membrane proteins and are in many cases required for their folding, stability, and function. The functional folding of membrane proteins in the presence of detergent micelles might therefore be quite different from the folding state of membrane proteins that are embedded in a lipid bilayer. For this reason, reconstitution of purified, detergent-solubilized membrane proteins into well-defined lipid bilayers has become a popular approach in membrane protein research.

One of the advantages of cell-free expression systems is the possibility of adding lipids to the reaction mix. This enables the co-translational insertion of the recombinant membrane protein into a well-defined lipid bilayer and thus eliminates the need for detergent solubilization and post-translational reconstitution of the target protein. Another advantage is that membrane proteins produced in L-CF mode are mainly integrated into the provided lipid bilayer in an inside-out orientation because the protein translation machinery is only present outside of the lipid vesicles (Schwarz et al., 2010). This results in more homogeneous membrane protein samples compared with the random orientation in classical post-translational reconstitution approaches.

In contrast to the more established P-CF and D-CF expression modes, the cell-free expression of membrane proteins in the presence of lipids is an emerging technique, and currently various strategies are being investigated. Different approaches have been successfully applied to the cell-free synthesis of membrane proteins in the presence of liposomes (Matsubayashi et al., 2014), nanodiscs (Proverbio et al., 2013), biological membrane environments (Wuu and Swartz, 2008), block copolymer membrane vesicles (proteopolymersomes; Nallani et al., 2011), and biomimetic lipid-detergent-based systems (Uhlemann et al., 2012). It should be realized, however, that most integral

membrane proteins do not insert spontaneously into the lipid membrane and require protein translocation components and molecular chaperones for efficient membrane integration (Martinez-Gil et al., 2011). Although chaperones or other translocation components can be added to the cell-free system to facilitate protein incorporation into the membrane (Kuruma et al., 2005; Roos et al., 2012), the reconstitution of membrane protein insertion pathways in an in vitro system still remains a challenging task. Furthermore, since efficient translocation is target-dependent, intensive protocol development for each new membrane protein target is essential. Important parameters that have an effect on cell-free expression levels and the efficiency of membrane integration are, for example, the type of lipid (e.g., charge and length of the alkyl chain), the lipid concentration, the type of bilayer (e.g., liposomes or nanodiscs), the DNA template concentration, and the incubation temperature (Nishiyama et al., 2006; Cappuccio et al., 2008; Katzen et al., 2008; Roos et al., 2013).

Because no routine strategy or universally applicable method is presently available for the cell-free production of membrane proteins in the presence of lipids, we recommend that the reader explore the abovementioned strategies to find optimal conditions for the L-CF expression of the particular membrane protein of interest.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2E****.*

Amino acid mix (4 mM each)

First, prepare single amino acid stock solutions of all 20 natural amino acids. Prepare for tyrosine a 20 mM stock solution and for the other 19 amino acids 100 mM stock solutions. Trp has to be dissolved in 100 mM HEPES, pH 8.0, whereas Asp, Cys, Glu, and Met have to be dissolved in 100 mM HEPES, pH 7.4. Adjust the pH of the HEPES buffers with potassium hydroxide (KOH). Dissolve all other amino acids in Milli-Q water. Sonication or heating to 60°C could improve solubility. However, some stocks (in particular Trp, Asp, Glu, Asn, Cys, and Tyr) will not dissolve completely and have to be handled as suspensions.

To obtain a mixture that contains 4 mM of each amino acid, combine 2 ml of each of the 100 mM amino acid stocks and 10 ml of the 20 mM tyrosine stock and make up to 50 ml with Milli-Q water. Store up to 12 months at −20°C.

Individual amino acids are available from Sigma.

NTP mix (33.3 mM ATP, 22.2 mM UTP, 22.2 mM GTP, 22.2 mM CTP)

Prepare stock solutions of ATP (133.2 mM), UTP, GTP and CTP (88.8 mM each) in Milli-Q water. Make the NTP mix by combining equal volumes of all four stock solutions. Store up to 12 months at −20°C.

NTPs are available from Sigma.

RCWMDE amino acid mix (16.7 mM each)

Prepare 100 mM stock solutions of amino acids Trp (in 100 mM HEPES, pH 8.0), Asp, Cys, Glu, Met (in 100 mM HEPES, pH 7.4), and Arg (in Milli-Q water). Mix equal volumes of the 100 mM stock solutions to obtain an RCWMDE amino acid mix that contains 16.7 mM of each of the six amino acids. Store up to 12 months at −20°C.

Individual amino acids are available from Sigma.

S12 extract buffer A

10 mM Tris·acetate, pH 8.2 (adjust pH with glacial acetic acid)
14 mM magnesium acetate
60 mM potassium glutamate
1 mM DTT
6.4 mM 2-mercaptoethanol
Prepare fresh

S12 extract buffer B

10 mM Tris·acetate, pH 8.2 (adjust pH with glacial acetic acid)
14 mM magnesium acetate
60 mM potassium glutamate
1 mM DTT
Prepare fresh

COMMENTARY

Background Information

G protein–coupled receptors (GPCRs) represent the largest family of membrane proteins in the human genome and the richest source of targets for the pharmaceutical industry. This is well illustrated by the observation that less than 10% of the three major GPCR subfamilies (class A, B, and C) already constitute the targets of approximately 30% of all drugs on the market (Harmar et al., 2009; Sharman et al., 2013). Despite their profound importance in human health and disease, very little is known about the detailed molecular mechanisms by which these membrane proteins are able to recognize their extracellular stimuli and transmit the associated messages through endogenous heterotrimeric G proteins. This lack of functional and structural data on GPCRs is a result of several challenges that are encountered during their production and isolation.

First, most GPCRs are present in native tissues at relatively low levels. Recombinant expression in a heterologous host is therefore the only practical way to obtain these receptors in milligram quantities required for structural studies. The most widely used expression systems for large-scale production of GPCRs include baculovirus-infected insect cells and human embryonic kidney cells (HEK293). Both systems have been successfully used to produce milligram quantities of purified, functional GPCRs for functional and structural studies (Andrell and Tate, 2013; Xiao et al., 2013). However, the overexpression and membrane insertion of GPCRs in these cellular systems frequently leads to increased basal signaling activity. This increased activity often interferes with cellular signaling pathways and introduces host cell toxicity, which results in slow growth rates and reduced expression yields. On top of this, overexpression in eukaryotic host cells is often accompanied by non-native or incomplete glycosylation of the recombinant GPCR. This is typically a result of overloading of the cellular glycosylation machinery, or occurs because the recombinant membrane protein is not compatible with the host-specific post-translational modification systems. Because the correct targeting of nascent GPCRs to the host cell membrane is usually dependent on specific glycosylation patterns that serve as sorting and trafficking signals, incomplete glycosylation of the recombinant GPCR often implies incorrect targeting of the receptor to the host cell membrane. Even when a recombinant membrane protein finally becomes efficiently inserted into the membrane, toxicity problems may arise due to pore-forming activities, overcrowding of the limited membrane space, or overloading of protein translocation systems.

Furthermore, the natural lipid environment of GPCRs limits the use of many standard techniques for studying soluble proteins, such as protein purification by FPLC, NMR, X-ray crystallography, and circular dichroism. Such methods are almost impossible to conduct in the native environment, and thus require extraction of the target protein from the host cell membrane. This is usually accomplished by adding a detergent that solubilizes the membrane and results in the formation of water-soluble protein-detergent complexes. Efficient solubilization, however, requires the use of harsh detergents that often lead to irreversible denaturation and aggregation of the membrane protein.

All of the challenges mentioned above are inherent problems associated with the

overexpression of integral membrane proteins in traditional cellular expression systems. Consequently, nearly all of these obstacles can be overcome by the elimination of a living host environment during protein overexpression. Such an environment can be readily achieved in cell-free expression reactions because the recombinant protein is produced in vitro using transcription and translation machinery extracted from cells. Since the cell-free reaction is not enclosed by membranes, problems with incorrect targeting and toxic effects of the recombinant membrane protein to the host cell physiology are minimized or even completely eliminated. The open environment of cell-free systems also offers the possibility to modify each parameter of the reaction depending on the target protein to be produced. Furthermore, compounds that may have a beneficial effect on the expression level, the solubility, or the stability of the recombinant protein can be added directly to the translation reaction. In this way, cell-free expression systems offer a variety of different strategies that can be followed for the overexpression of integral membrane proteins such as GPCRs. Mild detergents or lipid can be added directly to the synthesis reaction to provide a suitable hydrophobic environment for the nascent membrane proteins. This not only minimizes the aggregation of the synthesized membrane proteins, but also offers the opportunity to eliminate the often problematic detergent extraction step that is required to isolate the target protein from the host cell membrane. The detergent and lipid expression modes are unique for cell-free expression systems and cannot be obtained by traditional cell-based expression approaches.

Critical Parameters and Troubleshooting

Before starting to develop protocols for the purification and subsequent characterization of the membrane protein of interest, it is essential to achieve acceptable expression levels. Low expression levels will complicate the downstream process and can drastically affect the final purity and quality of the target membrane protein. In the worst case, the recovery may be even too low to provide information to assess a particular reaction condition, such as a detergent's suitability for the post- or co-translational solubilization of the recombinant protein.

A first critical parameter for successful cell-free expression of GPCRs is the design of the DNA template. In particular, the sequence of the first few nucleotides upstream of the start codon can have a profound effect on the translation efficiency. The addition of short N-terminal nucleotide sequences to the coding sequence of the target protein has proven to be a successful strategy to improve cell-free expression levels of difficult-to-express-proteins, including GPCRs (Ishihara et al., 2005; Ahn et al., 2007; Haberstock et al., 2012; Zhang et al., 2014). The expression tags are optimized to suppress secondary structure formation, which may prevent the initiation of translation. As secondary structures also depend on the target sequence, it is recommended to screen a set of expression tags for each particular target protein.

On top of the nucleotide sequences preceding the start codon, changes in codon usage can have a significant effect on translational efficiency. It may therefore be worthwhile to optimize the codon usage of your protein of interest, for example by gene synthesis, to replace the rare codons by more commonly used codons for the same residue.

Other critical parameters that have to be considered when optimizing expression levels are the final Mg^{2+} and K^{+} concentration in the cell-free reaction. Because suboptimal concentrations of Mg^{2+} and K^{+} can have a severe impact on protein synthesis, it is advisable to optimize the concentration of both ions for each new target and each new batch of cell extract.

A unique advantage of cell-free systems is the possibility to add detergents to the reaction, which allows the direct production of micelle-solubilized membrane proteins. A considerable variety of mild detergents are well-tolerated by cell-free systems, including the long-chain polyoxyethylene-alkyl-ethers (Brij series) and the steroid derivative digitonin. These detergents have been successfully used for the solubilization of different GPCRs, and should be the first choice when testing the soluble cell-free expression of a new receptor.

Although detergents are an indispensable tool for biochemical and structural studies of membrane proteins, they are frequently destabilizing and can lead to inactivation of the protein over time. To improve the stability and the yield of the expressed GPCR, stabilizing compounds can be added to the cell-free reaction. A frequently used stabilizer in GPCR research is cholesteryl hemisuccinate (CHS), a more water-soluble analog of cholesterol. The importance of cholesterol for GPCR function is supported by the fact that addition of cholesterol or its analogs can greatly increase the stability of GPCRs in solution

and can also improve crystal quality (Vukoti et al., 2012; Yeliseev, 2013). Recently reported crystal structures of the β_2-adrenergic receptor and the metabotropic glutamate receptor 1 have shown structural evidence for cholesterol binding sites, which has led to the identification of a consensus cholesterol binding motif in almost half of all family A GPCRs (Hanson et al., 2008; Wu and Sachs, 2014). Nevertheless, it should be noted that the effects of cholesterol on a particular receptor are unpredictable, and it has been demonstrated that certain GPCRs can be expressed as stable, ligand-binding and G-protein-activating receptors in *E. coli* membranes, which lack cholesterol (Oates and Watts, 2011).

Another strategy to stabilize GPCRs during expression and purification includes the addition of high-affinity ligands to the cell-free reaction and purification buffers. In this respect, it is noteworthy that so far no crystal structures of the ligand-free basal state of GPCRs have been determined (with rhodopsin being the only exception), demonstrating the importance of ligands in stabilizing the protein during isolation and crystallization.

Anticipated Results and Time Considerations

A crucial step in the development of a highly productive cell-free expression system is the preparation of a cell extract of good quality. The preparation of the crude cell extract takes approximately 2 days and yields around 10 ml extract from 1 liter of culture, which is sufficient to perform around 400 analytical-scale reactions with a reaction mix volume of 70 µl.

One of the advantages of cell-free systems is that the total process is less time- and effort-consuming when compared to the traditional cell-based expression methods. While the production of membrane proteins in traditional expression systems typically takes weeks to months, the optimized cell-free expression protocol can yield milligram amounts of purified membrane proteins within 1 week.

Although the protocols described in this unit should provide a good starting point for the cell-free expression of any GPCR (and by extension any type of membrane protein), optimal expression conditions are often target specific. Hence, it is recommended to optimize the most important parameters that can affect the expression level and stability of the overexpressed membrane protein. Factors that can influence the yield and quality of the recombinant protein are, for instance, the design of the DNA template, the final Mg^{2+} and K^+ ion concentrations in the reaction, and the type and concentration of the detergent that is used for co- or posttranslational solubilization. Optimization experiments can best be carried out in analytical-scale reactions (e.g., in 70-µl batch reactions) prior to scaling up. The optimization process can be performed within several weeks, but is dependent on the total number of parameters and DNA constructs that have to be tested. Typical results for the optimization of the final Mg^{2+} and K^+ concentrations in the cell-free reaction are given in Figure 29.14.1.

After target-specific optimization, the protocols described in this unit typically yield around 0.5 mg of soluble, purified GPCR per ml of reaction mix after cell-free expression in P- or D-CECF mode. Furthermore, analytical gel filtration and circular dichroism analyses indicate that the purified GPCRs are monodisperse in solution and adopt a mainly alpha-helical conformation (40% to 50%), suggesting that the receptors are correctly folded. Ligand interaction studies with the purified GPCRs gave further evidence of a functional folding of the cell-free produced receptors.

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