Chapter 2
Membrane Protein Quality Control in Cell-Free Expression Systems: Tools, Strategies and Case Studies

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2.1 Introduction

Cell-free (CF) expression has emerged in the last decade as an efficient and fast approach for the production of membrane proteins (MPs) of diverse topologies and origin. Its unique design as an open accessible reaction helps to eliminate several central bottlenecks known from conventional cell-based MP expression systems. In general, problems with cell physiology, expression regulation and cell culture are reduced. On the other hand, the high diversity of CF reaction conditions requests increased time investments in controlling MP quality, fine-tuning of reaction conditions and designing sample evaluation strategies. Poor MP sample quality can be the result if this important requirement is overseen.

CF reactions can basically be operated in two flavors, the single compartment batch configuration and the two-compartment continuous exchange (CECF) configuration (Kigawa and Yokoyama 1991; Spirin et al. 1988). The batch configuration is the method of choice in throughput applications using microplate devices and analytical scale reactions (Kai et al. 2013; Savage et al. 2007; Schwarz et al. 2010). Batch reaction times are limited to few hours with consequently lower yields of protein, although a number of modifications are possible in order to considerably improve efficiencies. Higher protein yields are typically obtained with the CECF configuration containing a reaction mixture (RM) compartment containing all the high molecular weight compounds such as ribosomes, DNA template and enzymes, and a feeding mixture (FM) compartment with a certain amount of precursors such as amino acids and nucleotides. Protocols for batch and CECF configurations are highly variable and among others, expression efficiencies depend on (1) precursor

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concentrations, (2) energy regeneration systems, (3) RM–FM volume ratios and (4) the implementation of repeated FM exchanges.

An array of new applications, modifications, and strategies for the CF production of MP samples has been developed within the last decade. In particular, the tools for the modulation of MP quality already during translation by CF reaction condition tuning have been widely expanded. We therefore provide a current view on options and perspectives for successful MP production and we summarize diverse strategies based on CF expression technologies.

2.2 Selecting the Background: Different Extract Sources

The origin of the CF extract is the first selection to be made by approaching MP expression. In particular, within the last decade, a considerable number of new extract sources covering eukaryotic as well as prokaryotic origins have been introduced (Table 2.1). Major selection criteria before starting a CF expression approach are usually (1) the required amount of synthesized recombinant protein, (2) to provide the most favorable background for promoting protein folding, (3) to increase the likeability of posttranslational modifications, (4) general handling issues, system availability and costs.

Expression efficiencies and other characteristics of the various systems still differ significantly and best compromises have to be found. While few micrograms of recombinant protein can usually be obtained in any system, the production of preparative scale levels approaching milligram yields out of 1 ml of RM is currently only routinely possible with extracts of *Escherichia coli* or wheat germs. Frequent limiting factors for protein production efficiency in cell extracts are high concentrations of endogenous degrading enzymes, poor synchronization of ribosome activity during cell growth, or stability problems of essential enzymes. It should be noted that extracts of cells showing even high expression activities *in vivo* such as yeasts might not be very efficient in CF expression. However, protocols in particular for the efficient CECF configuration are continuously being optimized and further potential for improved protein synthesis might exist. Most systems have now been adjusted as coupled transcription/translation systems including the efficient T7 promoter for protein production and accepting plasmid or linear DNA templates (Table 2.1). The addition of translation factors or considering specific template modifications might further be necessary depending on the selected system.

A critical issue is the availability of the different CF extracts. Most systems are available as standardized commercial kits, but quality optimization and specific applications often require the set up of individual expression reactions. The preparation protocols for the various cell extracts differ significantly with sometimes even high variations in extract batch quality (Table 2.1). For eukaryotic cell extract preparations, species possible to grow in defined cell cultures might be preferred or commercial sources might be considered. The relatively fast and efficient preparation protocol is a major advantage of using *E. coli* extracts. In addition, it is best

Table 2.1 CF extract sources

CF extract source	PTM[a]	cT7[b]	CECF[c]	Yield[d] (mg/ml)	MP[e]	Kit[f]	Protocol[g]
Eukaryotes							
Human HeLa S3 cells CHO	+	+	+	0.05–0.2 ≤0.01	NA	+	Mikami et al. (2006, 2008); Witherell et al. (2001)
Rabbit reticulocytes	+	+	−	≤0.01	+	+	Arduengo et al. (2007); Craig et al. (1992)
Wheat germ	+	+	+	≤2 (CECF) 0.1–0.4 (B)	+	+	Endo and Sawasaki (2005); Madin et al. (2000); Sawasaki et al. (2007); Takai et al. (2010)
Spodoptera frugiperda 9, 21	+	+	−	≤0.1	+	+	Ezure et al. (2007, 2010); Katzen and Kudlicki (2006); Stech et al. (2012)
Saccharomyces cerevisiae	−	−	−	0.05–0.07	NA	−	Wang (2006); Wang et al. (2008)
Leishmania tarantolae	−	+	−	≤0.2	NA	+	Kovtun et al. (2011)
Prokaryotes							
E. coli	−	+	+	≤5 (CECF) 0.5–1 (B)	+	+	Katzen et al. (2005); Schwarz et al. (2008)
Pseudomonas fluorescens (reaction at 8–30 °C)	−	−	−	≤0.1	NA	−	Nakashima and Tamura (2004)
E. coli–PURE (purified translation machinery)	−	+	+	≤0.5	+	+	Shimizu et al. (2001)
Thermus thermophilus (reaction at 37–65 °C)	−	−	+	≤0.06	NA	−	Uzawa et al. (2002); Zhou et al. (2012)
Thermococcus kodakaraensis (reaction at 40–80 °C)	−	−	−	≤0.007	NA	−	Endoh et al. (2008)

[a] Posttranslational modifications other than disulfide bridge formation reported
[b] Coupled transcription/translation protocol with T7 promoter described
[c] Protocols for the more efficient CECF configuration established
[d] Approximate range of protein yield in 1 ml of RM, *B* batch configuration, *CECF* continuous exchange configuration
[e] Expression of MPs reported; *NA* data not yet available
[f] Commercial reaction kits available
[g] Representative recent protocols for extract preparation

characterized and a large variety of compounds useful for reaction modifications is available. The vast majority of current data on MP production have thus been obtained with *E. coli* extracts.

Depending on the intended applications, the proper formation of posttranslational modifications can be a key issue for protein sample quality evaluation. Disulfide bridge formation may be triggered independently from extract origins by modulating the reducing conditions, e.g., by adding redox systems into the reaction, by supporting disulfide bridge formation with chaperones or by chemical pretreatment of extracts (Goerke and Swartz 2008, Kim and Swartz 2004, Yin and Swartz 2004). More complex modifications, such as glycosylation, lipidation, or phosphorylation, are so far only described from systems with eukaryotic extracts such as rabbit reticulocytes, insect cells, or wheat germ and at analytical scales (Table 2.1). Many modifications require supplements such as canine pancreas microsomes into the CF reaction. If modifying enzymes are provided, posttranslational modifications such as N-glycosylation appear to be possible even in extracts of *E. coli* (Guarino and DeLisa 2012). However, it might stay challenging to combine quality and homogeneity of posttranslational modifications with high-level expression purposes.

2.3 Basic Protocol Development: Improving CF Expression Efficiency

Complexity of MP production in CF systems is mainly reduced to the basic transcription/translation process. Coordination of pathways for trafficking or translocation as well as suppressing toxic effects are usually less relevant issues. Protein expression in most CF systems is controlled by the phage T7-RNA polymerase, and the corresponding regulatory promoter and terminator elements in addition to system specific enhancers have to be provided. However, other promoters could work as well. With *E. coli* extracts, derivatives of standard P*tac* promoters recognized by the endogenous *E. coli* RNA polymerase could give even relatively high expression levels (Shin and Noireaux 2010). DNA template constructs can be generated by overlap polymerase chain reaction (PCR) strategies and added as linear DNA fragment into the CF reaction (Ahn et al. 2005; Yabuki et al. 2007). Alternatively, plasmid DNA templates based on standard vectors such as, e.g., the pET or pIVEx series can be provided. DNA templates appear to be quite stable in CF reactions and final concentrations in between 2 and 10 ng/µl RM are already saturating (Haberstock et al. 2012).

Initial problems with low expression efficiency are mainly associated with the translation process. Adjusting the proper Mg^{2+} ion optimum is mandatory for each new target and suboptimal conditions can have severe impacts on protein production (Rath et al. 2011; Schwarz et al. 2007). Abundance of rare codons could further reduce protein expression and induce mis-incorporation of amino acids or even the premature termination of translation. Low protein yields are even more frequently caused by the formation of unfavorable secondary structures of the mRNA

involving the 5-prime end containing the translational initiation site. Modulating the nucleotide sequence of the 5-prime coding sequence can therefore be very efficient in order to improve expression (Ahn et al. 2007; Kralicek et al. 2011). A fast approach is the tag variation screen by analyzing the effects of a small number of short sequence-optimized expression tags (Haberstock et al. 2012). The tag variation constructs are generated by overlap PCR and the resulting products can directly be used as DNA templates in CF expression screens. The construction of large fusion proteins in order to improve expression is therefore usually not necessary. Expression monitoring can initially be performed via immunodetection by using C-terminal purification tags such as a poly$(His)_{10}$-tag as antigen. In an ideal template design, the coding sequence is therefore modified with a C-terminal purification/detection tag, and, if necessary, with a short N-terminal expression tag (Fig. 2.1). If translation can be addressed properly with the above mentioned procedures, the protein production in CF systems is usually very efficient. In expression screens comprising MP targets of diverse sizes, topologies, and functions, high success rates could be achieved (Schwarz et al. 2010; Savage et al. 2007; Langlais et al. 2007).

Expression monitoring by taking advantage of C-terminally attached derivatives of green fluorescent protein (GFP) could be useful for CF expression protocol development and fast protein quantification (Kai et al. 2013; Müller-Lucks et al. 2012; Nozawa et al. 2011; Roos et al. 2012). For MP expression, it must be considered that the folding of wild-type or red-shifted variants of GFP is hampered in the presence of most detergents (Roos et al. 2012). More resistant is the superfolder GFP derivative most likely due to its higher tolerance for chemical denaturants and its faster folding kinetics (Roos et al. 2012; Pedelacq et al. 2006). However, the folding of superfolder GFP might not correlate with the productive folding of the N-terminal target protein as it is speculated for other GFP derivatives (Pedelacq et al. 2006). Superfolder GFP might therefore only be considered as general expression monitor while fusions with other GFP derivatives may in addition also give some preliminary evidence of the target protein folding and quality.

2.4 Folded Precipitates: P-CF Expression

Depending on the strategy and choice of supplemented additives, several basic expression modes are possible for the CF production of MPs (Fig. 2.1). The selection of the expression mode may depend on the intended application of the MP sample, but it can also have drastic consequences on the resulting MP quality (Junge et al. 2010; Lyukmanova et al. 2012). An overview on the implementation of the different CF expression modes is given in Tables 2.2–2.4 and representative case studies published during the last decade are listed.

In absence of any provided hydrophobic environment, the freshly translated MPs instantly precipitate in the RM. Successful expression in this precipitate forming (P-CF) production mode can thus even be monitored by increased turbidity of the RM during incubation. Folded structures of such P-CF-generated MP precipitates

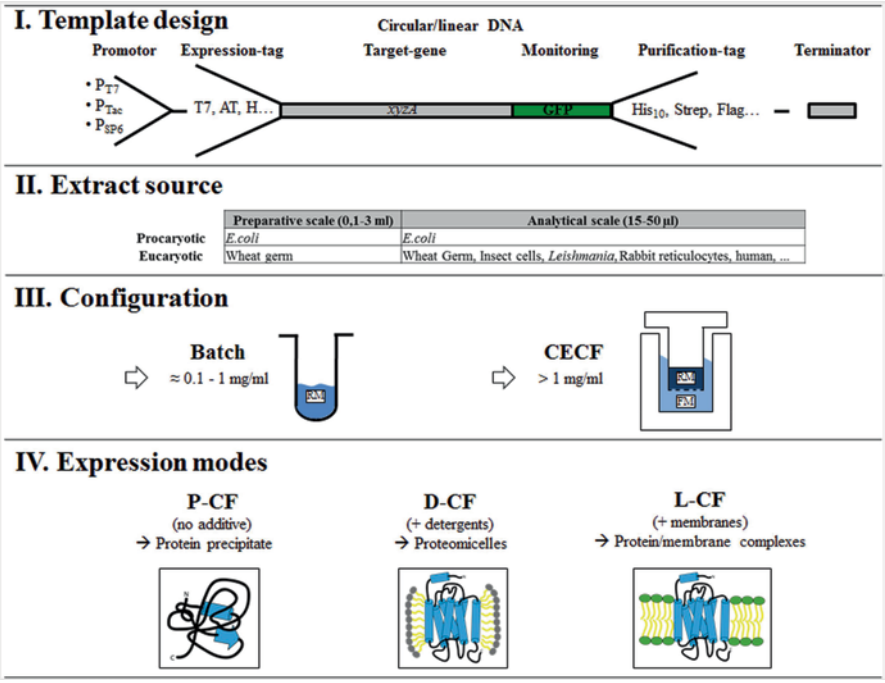

Fig. 2.1 Basic steps for the design of CF expression reactions

can be detected by solid state nuclear magnetic resonance (NMR) and resolubilized precipitates show significant structural overlaps with corresponding MP samples obtained after conventional *in vivo* production (Maslennikov et al. 2010). P-CF-expressed MPs can simply be harvested by centrifugation. The MP pellet is usually contaminated with a number of co-precipitated proteins from the extract. Washing with buffer containing mild detergents such as Brij derivatives can help to selectively reduce such contaminations. The MPs are then solubilized in buffer containing specific detergents. Best results are usually obtained with 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LMPG), 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LPPG), or sodium dodecyl sulfate (SDS; Table 2.2; Klammt et al. 2004; Klammt et al. 2012; Rath et al. 2011). Milder detergents such as n-dodecyl-phosphocholine (DPC) or n-dodecyl-b-D-maltoside (DDM), detergent cocktails or mixtures of detergents and lipids could further be useful depending on the MP target (Ma et al 2011).

Critical parameters for the resulting MP quality can be (1) detergent concentration and volume of the solubilization buffer, (2) temperature of solubilization, and (3) the subsequent exchange of the primary and usually relatively harsh solubilization detergent against secondary and considerably milder detergents, e.g., upon MP immobilization during affinity chromatography. Stabilization and high recovery of ligand binding active GPCRs could be obtained by this strategy (Junge et al. 2010;

Table 2.2 Case studies of P-CF-expressed MPs

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] yield[e]	Solubilization[f] (%)	Reference
Bs-MraY	36 (10)	Enzyme/+	iE [3]	Triton X-100 [2], DPC [2], DHPC [2], DDM [2], LS [0.8], LMPG [0.75], SDS [2]	Ma et al. (2011)
mAqp4-M23	30 (6)	Channel/+	iE [3]	Fos12 [1], DHPC [2], Fos16 [2], LMPG [2], LPPG [1]	Kai et al. (2010)
hETB, hNPY2/5 hMTNR1A/B hSS1/2, hV1BR hHRH1, hV2R rCRF	39–51	GPCR/-	iE [3]	e.g. LMPG [2]	Schneider et al. (2010)
hPS1-CTF	16 (3)	Protease/-	iE [3]	SDS [1–2]	Sobhanifar et al. (2010)
hCRFR1 mCRFR2β	47 (7) 49 (7)	GPCR/+	iE [3]	LMPG [2]/Nvoy	Klammt et al. (2011)
KvAP-VSD *A. pernix*	16.5 (4)	Channel/+	iE [2]	SDS [1]/DPC [0.2]	Lyukmanova et al. (2012)
hErbB3 (639–670)	5 (1)	Receptor/+	iE [3]	DPC	Mineev et al. (2011)
hErbB3 (632–675)	6 (1)	Receptor/+	iE [3]	SDS [1]	Khabibullina et al. (2010)
Ec-ArcB (1–115) Ec-QseC (1–185) Ec-KdpD (397–502)	11–21 (2–4)	Sensor/+	iE [3]	LMPG [5]	Maslennikov et al. (2010)
6 hMPs	~11 (2–3)	Unknown	iE [3]	LMPG	Klammt et al. (2012)
hLAPTM4A hCX32 hGLUT4 hVDAC1	28 (4) 32 (4) 55 (12) 36 (19)	Transporter Gap junction/+ Transporter Channel/+	iE [3]	Fos14	Nguyen et al. (2010)
F_1F_o-ATP synthase *C. thermarum*	542 (26)	Enzyme/+	iE [1]	DDM [2]	Matthies et al. (2011)
PorA/H *C. glutamicum*	5 (7)	Channel/+	iE [3]	LDAO [1], LPPG [1], LMPG [1], LMPC [1], Triton X-100 [1], DHPC [1], DPC [1], SDS [1]	Rath et al. (2011)
134 Ec-MPs	≤112 (≤15)	–	iE [3]	SDS [0.25], LPPG [0.25], LMPC [0.25], DPC [0.1]	Schwarz et al. (2010)

Table 2.2 (continued)

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] yield[e]	Solubilization[f] (%)	Reference
hETA/B	49/50 (7)	GPCR/+	iE [3]	LPPG [1], LMPC [1], SDS [1], Fos16 [1], Fos12 [1]	Junge et al. (2010)
Ec-EmrE	11 (4)	Transporter/+	iE [3]	DDM [2], DPC [1], LMPG	Klammt et al. (2004)
Ec-TehA	36 (10)				
Ec-SugE	11 (4)				
rOCT1/2	55 (12)	Transporter/+	iE [2]	LMPG [1]	Keller et al. (2008, 2011)
rOAT-1	60 (12)				
MPs of diverse origin	Diverse	Diverse	iE [3]	e.g. LMPG, DPC	Schwarz et al. (2007)
Proteorhodopsin	27 (7)	H^+-Pump/+	cE [2]	e.g. LMPG [0.01], LPPG [0.025]	Gourdon et al. (2008)
Bacteriorhodopsin *E. sibiricum*	28/(7)	H^+-Pump/+	iE	SDS, refolding	Shenkarev et al. (2013)
hH1R	56 (7)	GPCR/+	cE [1]	DDM [2]	Sansuk et al. (2008)
~120 MPs	10–30 (1–9)	Diverse	iE	nOG, DDM	Savage et al. (2007)
LH1-α-apoprotein *R. rubrum*	5 (1)	Light harvesting/+	cE [2]	Triton-X100 [0.5–2]	Shimada et al. (2004)
>100 hMPs	8–134	Diverse	cE/cWG	–	Langlais et al. (2007)

DDM n-dodecyl-β-D-maltoside, *DHPC* 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine, *DPC*=Fos12 n-dodecylphosphocholine, *Fos14* n-tetradecylphosphocholine, *Fos16* n-hexadecylphosphocholine, *LDAO* lauryldimethylamine oxide, *LMPC* 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac(1-choline)], *LMPG* 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)], *LPPG* 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)], *LS* n-lauroyl sarcosine, *nOG* n-octyl-β-D-glucopyranoside, *Nvoy* NV10 polymer, *SDS* sodium dodecylsulfate

[a] Approximate size, if documented, the origin of proteins is given in italics; *h* human; *m* murine; *r* rat; *Ec E. coli*, *Bs Bacillus subtilis*

[b] *TMH* Proposed number of transmembrane helices

[c] +: Quality analyzed by structural evaluation or functionality

[d] *iE* individual *E. coli* extract, *cE* commercial *E. coli* extract; *cWG* commercial wheat germ extract

[e] Approximate yields per one ml RM if documented in the corresponding references. 1: ≤0.1 mg/ml; 2: 0.1–1 mg/ml; 3: >1 mg/ml

[f] Detergents and concentrations used for posttranslational solubilization. Exchange into secondary detergents are indicated by slash. Concentrations are given if documented

Klammt et al. 2011). Solubilization of P-CF pellets is fast and usually complete after gentle shaking for approximately 1 h. It should be noted that pellets of CF-expressed soluble proteins cannot usually be solubilized by that procedure as they are much more unstructured. Consequently, MPs having excessive soluble domains could therefore resist solubilization out of P-CF pellets.

Selecting the P-CF expression mode is the fastest approach and usually routinely employed for the first level of MP expression protocol development in order to tune protein production up to the desired yields (Junge et al. 2011). Even complex MPs such as 12 transmembrane segment containing eukaryotic ion transporters or the 10 transmembrane segment containing MraY translocase have been functionally synthesized in the P-CF mode (Keller et al. 2008; Ma et al. 2011). The P-CF mode is furthermore excellent for screening MP libraries (Langlais et al. 2007; Savage et al. 2007; Schwarz et al. 2010) and for the production of MP samples for structural analysis by NMR (Klammt et al. 2004; Maslennikov et al. 2010; Rath et al. 2011; Sobhanifar et al. 2010).

2.5 Production of Proteomicelles: D-CF Expression

CF expression systems can tolerate a considerable number of supplied hydrophobic compounds, while certain variations in between the different extract sources exist. The CF expression in the presence of detergents above their critical micellar concentration (CMC) can result into the co-translational solubilization of the expressed MPs and into the instant formation of proteomicelles (Fig. 2.1).

Extensive evaluation of detergent tolerance has been performed with *E. coli* extracts (Blesneac et al. 2012; Gourdon et al. 2008; Klammt et al. 2005; Lyukmanova et al. 2012) as well as with wheat germ extract systems (Beebe et al. 2011; Genji et al. 2010; Kaiser et al. 2008; Periasamy et al. 2013). As primary compounds of choice, long-chain polyoxyethylene-alkyl-ethers such as Brij35, Brij58, Brij78 or Brij98, and the steroid-derivative digitonin have been determined (Table 2.3). These detergents have been successfully used for the solubilization of different G protein-coupled receptors (GPCRs) as well as of prokaryotic MPs (Table 2.3). Commonly employed detergents for the extraction of MPs out of native membranes such as DPC, the alkyl-glucoside n-dodecyl-β-D-maltoside (DDM), or n-octyl-β-D-glucopyranoside (β-OG) are too harsh or only tolerated at lower concentrations. However, the tolerance can sometimes be increased if critical detergents are provided as mixed micelles together with other detergents, e.g., CHAPS together with Fos-choline derivatives (Genji et al. 2010). It might generally be advantageous to combine the provided detergent micelles with some small amounts of lipids in case the translated MPs require interaction with some lipids for stabilization (Arslan Yildiz et al. 2013; Müller-Lücks et al. 2013; Nozawa et al. 2007). For the expression of mitochondrial carrier proteins, the addition of some cardiolipin together with fluorinated surfactants or Brij35 detergent had significant beneficial effects, whereas cardiolipin had negative effects in combination with the detergent Brij58

Table 2.3 Case studies of D-CF-expressed MPs

Protein[a]	Size [kDa] (TMH[b])	Type/assay[c]	System[d] Yield[e]	Detergent[f]	Reference
Pores and Channels					
Aqp3	32 (6)	Porin/+	iE [3]	Brij98 + Ec polar lipids	Müller-Lucks et al. (2013)
Cx32	32 (4)	Channel/+	cE [2]	Brij35	Nguyen et al. (2010)
VDAC	36 (1 + 13ß)				
hVDAC1	35 (13ß)	Channel/+	cE [1]	DDM, Fos12	Deniaud et al. (2010)
mAqp4	30 (6)	Porin/+	iE [3]	Brij35, Digitonin	Kai et al. (2010)
Ec-MscL	15 (2)	Channel/+	cE [3]	Triton X-100	Berrier et al. (2004); Abdine et al. (2010)
OEP24	24 (12ß)	Channel/+	cE [2]	DDM	Liguori et al. (2010)
PorA/H *C. glutamicum*	5	Channel/+	iE [2]	Brij72	Rath et al. (2011)
hERG	25 (6)	Channel/+	iE [2]	Brij78 + soybean PC	Arslan Yildiz et al. (2013)
Transporters and pumps					
UCP1	30–35 (6)	Carrier/+	cE [2]	Brij35/58, DDM, digitonin, fluorinated surfactants + cardiolipin	Blesneac et al. (2012)
Ec-EmrE	12 (4)	Transporter/+	cE [3]	DDM	Elbaz et al. (2004)
AtPPT1, OpPPT1/2/3	30 (8)	Transporter/+	cE	Brij35 + Asolectin	Nozawa et al. (2007)
Bacteriorhodopsin	28 (7)	H^+-Pump/+	cE [2]	NaPol	Bazzacco et al. (2012)
Bacteriorhodopsin	28 (7)	H^+-Pump/+	WG	Chaps + Fos12, Fos14	Genji et al. (2010)
Bacteriorhodopsin	28 (7)	H^+-Pump/+	WG	Chaps, Fos12	Beebe et al. (2011)
Ec-Tsx	34 (12ß)	Transporter/–	iE [3]	Brij78	Klammt et al. (2005)
Receptors					
Dopamine D2	50 (7)	GPCR/+	iE WG	–	Basu et al. (2013)
hTAAR-T4L	45 (7)	GPCR/+	iE [2]	Brij35	Wang et al. (2013)
hETA, hETB	~45 (7)	GPCR/+	iE [3]	Brij35/78	Junge et al. (2010)
Olfactory Receptors, hFPR3, hVN1R1, hVN1R5	~30 (7)	GPCR/+	cE [2]	Brij35, peptide surfactants	Corin et al. (2011)
Cytokinin Receptor CRE1/AHK4	37 (2)	Receptor/+	iE [3]	Brij58/78	Wulfetange et al. (2011)
CpxA	50 (2)	Receptor/+	cE [3]	Brij35	Miot and Betton (2011)

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