Novel cell-free expression system for synthesis of proteins used in structural analyses

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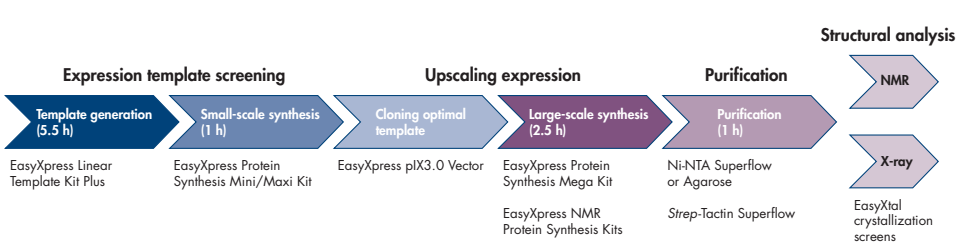
Introduction — Producing proteins for NMR and X-ray crystallography

Cell-free expression systems provide an attractive alternative to conventional in vivo methods by dramatically reducing the time required to obtain proteins and allowing easy incorporation of labeled amino acids without cytotoxicity (Se-Met) or amino acid metabolism (isotope-scrambling) problems (1).

They also enable fast identification of an optimal expression construct (2) and offer the possibility of efficiently producing proteins that are poorly expressed in in vivo systems.

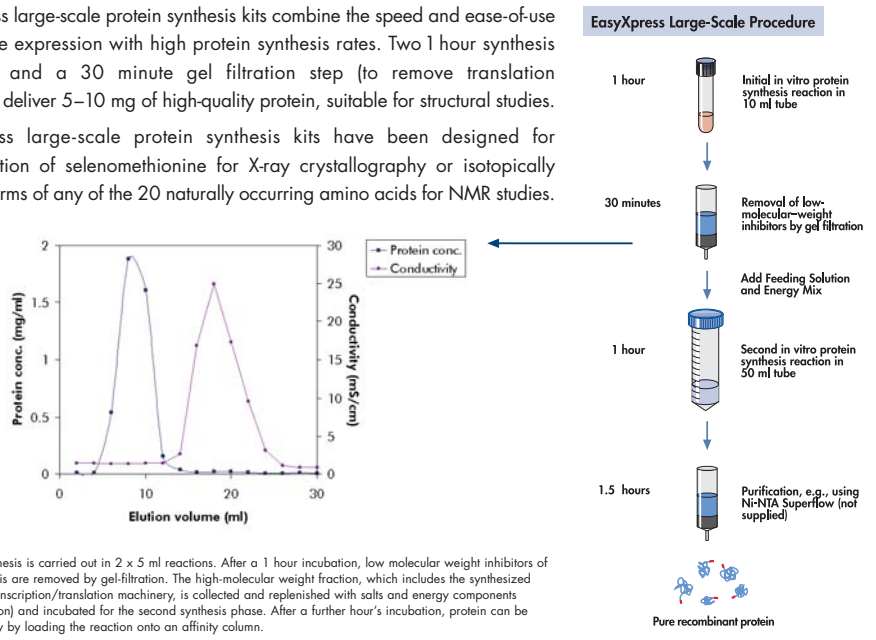
Adding an affinity tag to the expression construct and using large-scale reactions enables sufficient Se-Met or SI-labeled protein for a thorough structural determination to be synthesized and purified to homogeneity in a single working day.



EasyXpress Large-Scale Kits — Principle and procedure

EasyXpress large-scale protein synthesis kits combine the speed and ease-of-use of cell-free expression with high protein synthesis rates. Two 1 hour synthesis reactions and a 30 minute gel filtration step (to remove translation inhibitors) deliver 5–10 mg of high-quality protein, suitable for structural studies.

EasyXpress large-scale protein synthesis kits have been designed for incorporation of selenomethionine for X-ray crystallography or isotopically labeled forms of any of the 20 naturally occurring amino acids for NMR studies.



The initial synthesis is carried out in 2 x 5 ml reactions. After a 1 hour incubation, low molecular weight inhibitors of protein synthesis are removed by gel-filtration. The high-molecular weight fraction, which includes the synthesized protein and transcription/translation machinery, is collected and replenished with salts and energy components (Feeding solution) and incubated for the second synthesis phase. After a further hour's incubation, protein can be purified directly by loading the reaction onto an affinity column.

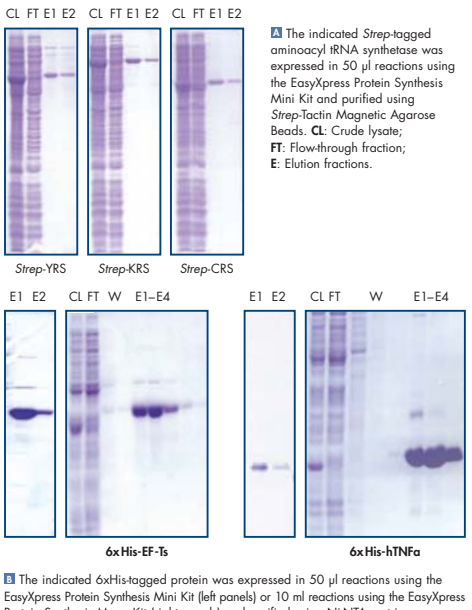
Upscaling in vitro protein expression

Before large-scale (10 ml) synthesis reactions, small-scale (50 µl) screening experiments are usually performed to determine the optimal expression construct. Factors that can significantly influence expression rate and yields are protein solubility, codon usage, secondary structures in the mRNA 5' UTR, affinity tag position, and expression vector.

Yield per ml reaction volume increases during upscaling by a factor of 1.5–2 (compare mg/ml columns). The presented examples show that scaling up in vitro protein expression works reliably and that EasyXpress large-scale reactions deliver 5–10 mg of protein, sufficient for most structural genomics studies.

Protein	Origin	Vector	Affinity tag (terminal)	Yield per 50 µl small-scale reaction	Yield per 10 ml large-scale reaction
Ubiquitin	Human	pIX2.0	*Strep* II (C)	21.1 µg (0.4 mg/ml)	6.1 mg (0.6 mg/ml)
FABP	Human liver	pIX3.0	6xHis (N)	8.3 µg (0.17 mg/ml)	5.3 mg (0.5 mg/ml)
FABP	Bovine	pIX2.0	6xHis (N)	21.0 µg (0.5 mg/ml)	8.5 mg (0.9 mg/ml)
TFIIB	Human	pET14b	6xHis (N)	10.1 µg (0.2 mg/ml)	5.0 mg (0.5 mg/ml)
TNFα	Human	TAGZyme pQE-2	6xHis (N)	15.0 µg (0.3 mg/ml)	5.3 mg (0.5 mg/ml)
Taq	*T. aquaticus*	pET30b	6xHis (N)	8.0 µg (0.16 mg/ml)	2.9 mg (0.3 mg/ml)
EF-Ts	*E. coli*	pIX2.0	6xHis (N)	35.1 µg (0.7 mg/ml)	8.5 mg (0.9 mg/ml)
FtsY-NG	*E. coli*	pET9d	6xHis (C)	6.2 µg (0.13 mg/ml)	4.4 mg (0.4 mg/ml)
KRS*	*E. coli*	pIX2.0	*Strep* II (N)	15.0 µg (0.3 mg/ml)	ongoing
CRS†	*E. coli*	pIX2.0	*Strep* II (N)	6.4 µg (0.14 mg/ml)	ongoing
YRS‡	*E. coli*	pIX2.0	*Strep* II (N)	7.5 µg (0.15 mg/ml)	ongoing

*Lysyl-tRNA synthetase, †Cysteinyl-tRNA synthetase, ‡Tyrosinyl-tRNA synthetase

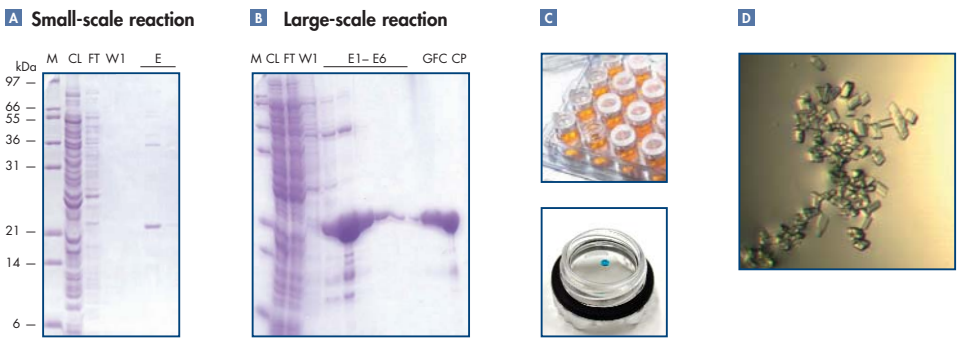


A The indicated *Strep*-tagged aminoacyl tRNA synthetase was expressed in 50 µl reactions using the EasyXpress Protein Synthesis Mini Kit and purified using *Strep*-Tactin Magnetic Agarose Beads. **CL**: Crude lysate; **FT**: Flow-through fraction; **E**: Elution fractions.

B The indicated 6xHis-tagged protein was expressed in 50 µl reactions using the EasyXpress Protein Synthesis Mini Kit (left panels) or 10 ml reactions using the EasyXpress Protein Synthesis Mega Kit (right panels) and purified using Ni-NTA matrices. **CL**: Crude lysate **FT**: Flow-through fraction; **W**: Wash fractions; **E**: Elution fractions.

Producing protein for X-ray crystallographic analysis

After evaluation of the expression template in a small-scale reaction the N-terminal GTP-binding domain (NG) of FtsY, the *E. coli* homolog of signal recognition particle receptor α (3), was synthesized in a 10 ml EasyXpress Protein Synthesis Mega Kit reaction containing selenomethionine (Se-Met). His-tagged FtsY-NG was purified using Ni-NTA Superflow and high-resolution gel filtration. After purification, protein fractions were pooled and concentrated by ultrafiltration. Highly concentrated protein (7.3 mg/ml) of high purity (97.6%, Agilent Bioanalyzer 2100) was used for crystallization trials using EasyXtal protein crystallization products.

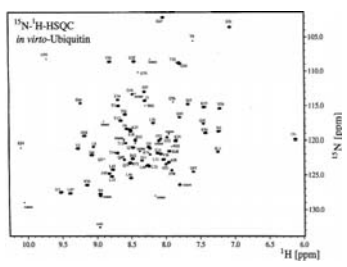


A After evaluation of the expression template in a small-scale reaction, the N-terminal GTP-binding domain (NG) of FtsY, the *E. coli* homolog of signal recognition particle receptor α (3), was synthesized in a 10 ml EasyXpress Protein Synthesis Mega Kit reaction B His-tagged FtsY-NG was purified using Ni-NTA Superflow and gel filtration. **M**: markers; **C**: crude lysate; **F**: flow-through; **W**: wash fractions; **E**: elution fractions; **G**: gel-filtration column pool; **C**: concentrated protein. After purification, protein fractions were pooled and concentrated by ultrafiltration. 4.4 mg of highly pure concentrated protein (7.3 mg/ml, 97.6% pure [Agilent Bioanalyzer 2100]) was obtained for crystallization. FtsY-NG was crystallized in the absence of GTP using the EasyXtal Classics Screening Suite C by hanging-drop vapor diffusion method in pre-filled crystallization plates. D Crystals were obtained after 7 days in $CaCl_2$/Na-acetate/MPD (condition no. 15). Only micro crystals were obtained under previously published crystallization conditions (3).

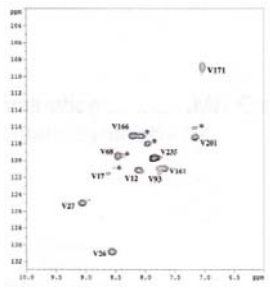
Protein structure analysis by NMR

NMR spectroscopy is an alternative to X-ray crystallography for determining protein structures. The method is based on incorporation of amino acids labeled with stable heavy isotopes (e.g., D, ^{13}C, ^{15}N). The table opposite gives an overview of labeling methods. In vitro expression has significant advantages over in vivo expression for generation of Stable-Isotope (SI) labeled proteins by allowing both uniform and reliable amino-acid specific SI-labeling.

Labeling method	Comments	In vivo expression	In vitro expression
Uniform — all amino acids labeled	Most widely used method	Cell growth using ^{13}C-glucose, $^{15}NH_4^+$, or D_2O	Uniformly labeled amino acid mix
Amino-acid specific — e.g., all valines	In vivo labeling can be problematic (isotope-scrambling)	Cell growth using synthetic medium including single labeled amino acid	Amino acid mix including single labeled amino acid
Site-specific — labeling individual residues	Labeling residues at active sites enables drug screening by NMR	Accomplished by use of unnatural codon and synthetic tRNA	Accomplished by use of amber codon and synthetic tRNA



Strep-tagged human ubiquitin was expressed using the EasyXpress Protein Synthesis NMR Kit in the presence of an amino acid mix for uniform labeling, purified by single-step *Strep*-Tactin affinity chromatography, and subjected to 2D NMR analysis. The ^{1}H–^{15}N heteronuclear single quantum correlation (HSQC) spectrum showed the expected signals.



The N-terminal domain of a human protein was expressed using the EasyXpress NMR Protein Synthesis NMR Kit using ^{15}N-valine to specifically label valine residues. 2.5 mg of ^{15}N-valine labeled protein was used in 2D NMR analysis. All valine residues contained in the protein could be detected in the ^{1}H–^{15}N HSQC spectrum.

Conclusions

We have developed an in vitro expression system, which, following a small-scale template evaluation step, can be used for synthesis of milligram amounts of functional proteins, and have demonstrated its application to X-ray crystallographic and NMR spectroscopic structural analysis of prokaryotic and eukaryotic proteins. In vitro synthesis offers several advantages over traditional in vivo methods, including:

- **Fast expression template screening** — gene to protein within a single working day
- **Fast upscaling of expression** — milligrams of purified protein within 4 hours
- **Label incorporation uncompromised by host cell physiology** — high-quality protein

References
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(3) Montoya, G., et al. (1997). Crystal structure of the NG domain from the signal recognition particle receptor FtsY. Nature **385**, 365.

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