Center for Eukaryotic Structural Genomics

Protein Structure Initiative

Use of Cell-Free Protein Production Platform for X-ray Crystallography

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Abstract

The Center for Eukaryotic Structural Genomics (CESG) has been working to further develop our *in vitro* transcription-wheat germ cell-free translation system for the production of proteins for structural studies. To date, sixteen NMR structures have been determined at CESG from protein produced by our cell-free platform. More recently, attention has been turned to the use of this platform for X-ray crystallography. The first structures from CESG to be determined from cell-free sources are two crystal forms of agmatine iminohydrolase, one in complex with a ligand, thereby extending earlier work on this protein by CESG, and the structure of a complex of two human proteins involved in DNA replication produced by simultaneous translation in the cell-free system. The creation of this complex represents a control experiment, following earlier work on a similar complex by Deng et al.(2) Two other novel target proteins, human cystatin-M and a mammalian DCN1-like protein 2 homolog from the thermophilic algae *Galderia sulphuraria* have been crystallized. The DCN1 homolog crystals diffract to a resolution of 1.2 Å and its structure determination has recently been completed. Crystals of cystatin-M are in our diffraction screening process. Incorporation of selenomethionine into proteins produced with the cell-free methods is as straightforward as substituting selenomethionine for methionine in the reaction mixture. There are no toxic side effects of selenomethionine, and incorporation is essentially 100 percent. These results clearly establish that the method is capable of providing support for structural genomics and structural biology research projects.

CESG's partnership with CellFree Sciences Co., Ltd. (Yokohama, Japan) and with Prof. Yaeta Endo has steadily improved the capabilities of wheat-germ based protein production systems during PSI-1 and PSI-2 (1). The costs can be made quite reasonable. While the reactions can be carried out on the bench top, robotic support allows for high-throughput screening and also improves reproducibility due to standardization of liquid handling and timing of events (Table 1) . Small-scale screening on the 1-5 microgram scale can be performed on the GenDecoder instrument for a cost of about $1/sample (not including DNA production costs). At the next scale-up level, the procedure from DNA to purified protein can be carried out in automated fashion on the Protemist DT-II bench-top robot in sufficient quantities for small-scale crystallization screening in favorable circumstances when all channels are dedicated to one protein. Scaling to the next level, the Protemist series and Protemist XE are capable of larger scale productions but without the protein purification capability.

Table 1. Cell-Free Protein Production Robotics capabilities available to the NP Partnership.

	GenDecoder1000	Protemist DT-II	Protemist100	ProtemistXE (prototype)
				
Typical Use	HTP Screening	Screening; Characterization	Characterization; Production	Production
Format	96 well; 4x96 well	24 well; 6 well	8 tubes	1 reaction
Total Reaction Volume	14.4-19.2 mL	33.6 mL; 24 mL	32 mL	10-20 mL
Reaction Time	24 h	14 to 24 h	18 h	12 to 48 h
Automated Transcription[a]	Yes	Yes	Yes	No
Automated Translation	Yes	Yes	Yes	Yes
Automated Purification[b]	No	Yes	No	No
Yield Per Reaction[c]	1 to 5 µg	50 µg to 1 mg	0.5 to 10 mg	1 to 70 mg

[a]Reaction initiated by provision of purified plasmid DNA. [b]Protemist-DTII provides automated purification in the 6 well mode. [c]Based on CESG results with expressed proteins; 70% of proteins tested fall inside these expression yield thresholds.

Results and Discussion

CESG has begun to use cell-free translation to solve structures using X-ray crystallography, and four have been completed since April 2009. Proof-of-concept studies were carried out with agmatine iminohydrolase. A purified sample of 7.7 mg was obtained from 6 mL of the cell-free extract 2240H (1.3 mg/ml extract, a better than average yield for cell-free translation; our cost $2700). For comparison, 110 mg was obtained from 2000 mL of *E. coli* culture, a considerably better than average yield for a eukaryotic protein in *E. coli*.) The selenomethionyl incorporation determined by mass spectrometry was 99% in the cell-free sample, exactly matching the composition of the added amino acid. The deposited structure of the native protein (PDB 3H7C, see below) from cell-free translation had a resolution of 1.5 Å, comparable to the cell-based structure (PDB 1VKP). A functional follow-up study using the same batch of cell-free protein yielded a structure of a covalent intermediate (PDB 3H7K, see below). As another test, CESG chose a heterodimer complex originally prepared by other from *E. coli* with a yield of ~ 0.02 mg/mL of culture. Their preparation required a complicated purification process; some crystals took as long as 1 year to grow; and the structure was solved to 2.5 Å resolution (2). In our cell-free translation system, the intact protein complex was obtained by simply adding two mRNAs to the translation reaction, with a protein yield of 2.5 mg from 10 mL of extract (WEPRO 2210H, $4500).

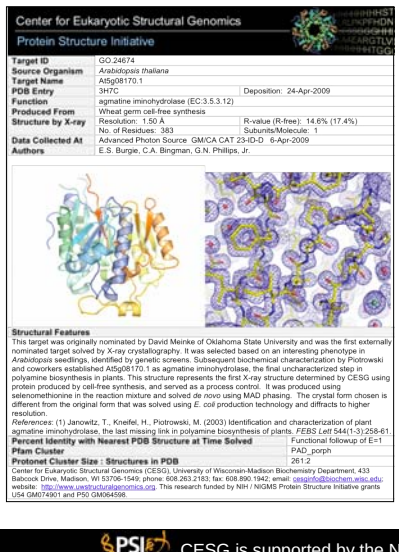
The Sesame LIMS shows that the total elapsed time from availability of the two cloned genes to solved structure was 137 days. Crystals were obtained in different conditions than the previous work and had a different space group. The structure obtained from cell-free translation (PDB 3KDF, see below) had a better resolution of 2 Å. An important feature of this work was that the open nature of cell-free translation was successfully exploited to assemble target solved by X-ray crystallography. A third PDB entry, 3KEV, represents our first *de novo* cell-free structure (see below for details). The Protemist XE was used with 5 mL of WEPRO 8240H extract (our cost $4000), and the yield of purified protein was 4.2 mg. The cell-free capability has also been used in functional studies of the human desaturase-cytb$_5$ complex, and is incorporated into our proposed combinatorial discovery efforts with other membrane proteins. The system has also been used to produce other membrane proteins using liposomes as the initial repository of the polypeptide chains (3). As a control for the structure determination process, bacteriorhodopsin has been produced in milligram quantities and crystallizations are underway following earlier published work by others.

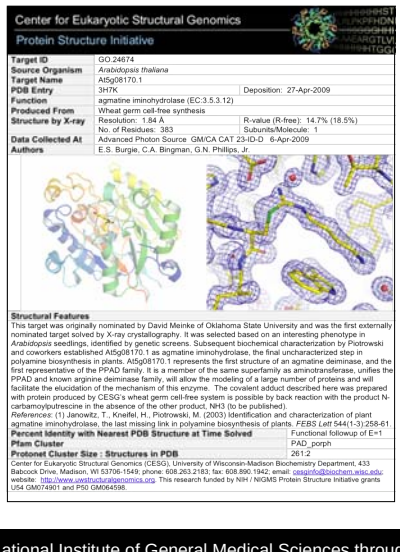
Advantages of the system are that it sometimes produces soluble protein when other systems fail and that it involves lower labor costs. (Fifteen project personnel were involved in the *E. coli* effort vs. eight project personnel are involved in the cell-free effort.) Disadvantages are a steep learning curve and the supply cost. The key determinant of the supply cost of this cell free system is the amount of wheat germ extract committed to a trial. As in other expression systems, many times proteins are expressed only in insoluble forms, and small-scale screening trials are advised. When producing soluble proteins, the yield per mL of wheat germ extract is variable, but an upper limit (for GFP) is about 4 mg per mL of extract in the Protemist 100. More typical numbers when the process works are 0.1-0.5 mg of purified protein per mL of extract. In the Protemist XE, which has been optimized for larger scale production using a more stringently prepared wheat germ extract, the yield per mL of extract is increased four-fold.

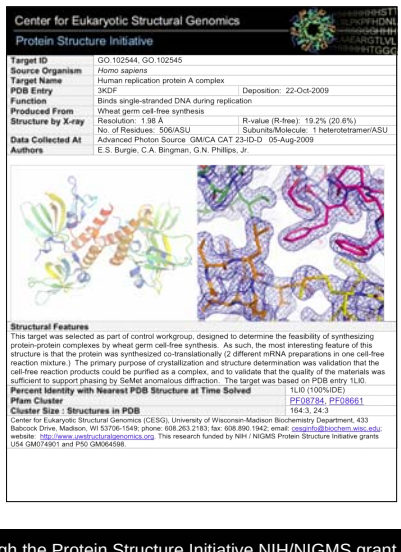
References

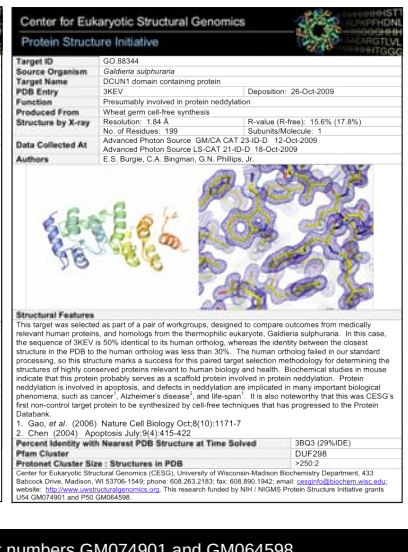
1. Tyler *et al.* Comparison of cell-based and cell-free protocols for producing target proteins from the *Arabidopsis thaliana* genome for structural studies. *Proteins* 2005 39:633.
2. Deng *et al*. Structure of the full-length human RPAA14/32 complex gives insights into the mechanism of DNA binding and complex formation. *JMB* 2007, 474:865.
3. Goren, *et al*. Cell-free translation of integral membrane proteins into unilamelar liposomes. *Methods Enz* 2009, 463:647.

Note: Emerald BioSystems is now the US distributor of CellFree Sciences Products. (Disclosure: No financial consideration has been received by the authors or CESG from Emerald BioSystems. A collaboration with CellFree Sciences Co., Ltd. has provided instruments at discounted prices and wheat germ extract and other supplies to CESG at substantial discount; authors have received no financial remuneration.)









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