Center for Eukaryotic Structural Genomics

Protein Structure Initiative

High-throughput automated platform for NMR-based structural proteomics

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Abstract

The Center for Eukaryotic Structural Genomics (CESG), in cooperation with Ehime University, (Matsuyama, Japan), and the Cell-Free Sciences Co., Ltd. (Yokohama, Japan), has developed an automated platform for NMR-based structural proteomics that employs wheat germ extracts for cell-free production of labeled protein. The platform utilizes a single construct for all targets without any redesign of the DNA or RNA; it thus offers advantages over commercial cell-free methods utilizing *E. coli* extracts that require multiple constructs or redesign of the ORF. The protein production and labeling protocol is no more costly than *E. coli* cell-based approaches, is robust and scalable for high-throughput applications, and has been used in our Center to screen eukaryotic ORFs from the *Arabidopsis thaliana* and human genomes and for the determination of NMR structures. With the recent addition of a Cell-Free Sciences GeneDecoder 1000™ robotic system, CESG is able to carry out as many as 384 small scale (50 µl each) screening reactions per week, and with the use of a Cell-Free Sciences Protemist™ robotic system, CESG can carry out 16 production-scale (4 ml each) reactions per week. We will present our latest results and experience in utilizing this automated platform technology to screen targets for expression and solubility and to produce stable-isotope labeled samples for NMR structure determinations.

Rationale for exploring this technology

Structural proteomics projects require the expression and purification of thousands of proteins and/or protein fragments. The ability to obtain labeled proteins is an essential requirement for rapid progress in structure determinations. Critical examples are (1) the production of U-^{15}N labeled protein to assess the foldedness and aggregation state, (2) the production of U-^{13}C;^{15}N or selectively labeled proteins for NMR structure determinations, and (3) the production of Se-Met labeled protein for phase determination in X-ray studies. The successful implementation of cell-free protein expression may minimize problems in cell harvesting, cell lysis, and pre-column manipulations. Moreover, this approach may simplify purification, because the protein of interest is isolated from a smaller set of contaminants. Cell-free systems may also permit labeling strategies that cannot be achieved in whole cell systems, while potentially providing a substantial economy in the labeled material required to produce a target protein. This promise warrants serious consideration of how cell-free translation can contribute to high-throughput structural biology.

Advantages of the wheat germ cell-free protein production

- Supports rapid and efficient screening (supported by robotics)
- Requires smaller volumes (avoids lengthy concentration steps in protein purification)
- Labeled proteins can be prepared rapidly (in 1-2 days) to meet the needs of structural biologists
- Supports labeling strategies that are not practical for proteins produced from bacterial cells (no label scrambling)
- Supports the production of eukaryotic N-terminal (His)$_6$ proteins (previous experience showed that these were not produced successfully from *E. coli* cells)

Disadvantages of the wheat germ cell-free protein production

- Reagent intensive
- Currently not compatible with Gateway Cloning Technology used in the *E. coli* cell-based pipeline of the CESG project

CESG's wheat germ cell-free protein expression project involves two groups

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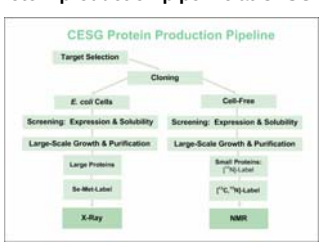
- Screening of potential targets from eukaryotic genomes for suitability for structural studies
- Production of labeled proteins on the scale of several milligrams
- Assessment of this approach for high-throughput structure determination
- Improvement of the technology through its use in a production environment

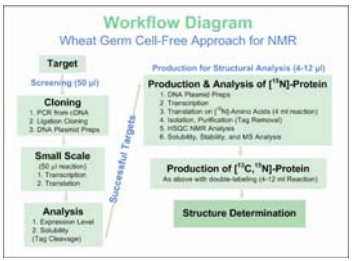
Ehime University and Cell-Free Sciences Co., Ltd.

- Wheat germ extract production
- Enabling methodology
- Robotics

Protein production pipeline at CESG



Wheat germ cell-free approach for NMR



Expression vectors

N-(His)$_6$ – pEU Vector - uncleavable

- SP6 - Ω - (His)$_6$ - MCS - AA added to the N-terminus: MG(H)$_6$LE

N-(GST) – pEU Vector – cleavable with PreScission™ Protease

-SP6 - Ω - GST - PreScission - MCS –

-AA added to the N-terminus : GPLE

Expression and solubility screening of ORFs with N-terminal (His)$_6$ tag

Source	Expression +		Expression -		Solubility +		Solubility -	
Arabidopsis	182	77%	55	23%	94	52%	88	48%
Human	66	78%	19	22%	28	42%	38	58%
Mouse	47	59%	33	41%	28	60%	19	40%
Total	295	73%	107	27%	150	51%	145	49%

"Solubility +" indicates that target protein is at least 50% soluble

Overall success rate for producing highly-soluble protein with N-terminal (His)$_6$ tag:

Arabidopsis: 40% Human: 33% Mouse: 36%

Expression and solubility screening of ORFs with N-terminal GST tag

Source	Expression +		Expression -		Solubility +		Solubility -	
Arabidopsis	86	79%	23	21%	56	65%	30	35%

"Solubility +" indicates that target protein is at least 50% soluble

55 / 56 target proteins remained > 98% soluble after tag cleavage

Overall success rate in expressing soluble protein by the cleavable GST route was 49%

Structural investigations

Source	Low Yield / No HSQC	Higher Yield / HSQC possible	Folded / Non-Aggregating	Unsuitable for NMR structure	# Structures
Arabidopsis	51 50%	51 50%	23 45%	28 55%	5*
Human	10 30%	23 70%	9 40%	14 60%	1**
Mouse	1 8%	11 92%	2 18%	9 82%	1***

One *Arabidopsis* structure is in progress; ** one human structure is in progress; *** one mouse structure is in progress.

Average yield for proteins used in structural investigations – 0.5 mg/ml

Comparison of target progress through three CESG pipeline protocols

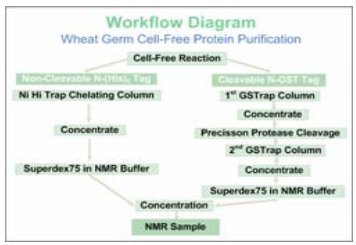
Ultimate step reached	Number of targets reaching a given ultimate step: *E. coli* MBP-tag All	< 21	> 21	Cell-free His-tag All	< 21	> 21	Cell-free GST-tag All	< 21	> 21	Cell-free (best of both tags) All	< 21	> 21	Best of all protocols: *E. coli* and cell-free All	< 21	> 21
Sequence - (Seq)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Destination - (D)	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
E = Expression - (E)	39	19	20	14	6	8	12	2	10	6	1	5	2	0	2
Solubility - (S)	2	1	1	32	13	19	39	22	17	32	16	16	19	9	9
Cleavage - (C)	16	10	6	0	0	0	8	1	7	0	0	0	7	5	1
Purification - (P)	4	1	3	5	4	1	5	0	5	4	3	1	8	2	4
Low yield (L) -	16	1	15	28	11	14	22	8	14	24	7	17	27	8	19
NMR sample prep - (N)	0	0	0	2	1	1	0	0	0	2	1	1	1	0	1
HSQC acquired	18	12	6	16	10	6	21	13	8	28	17	11	35	20	15
HSQC -	11	6	5	8	4	4	11	7	4	17	10	7	24	13	11
HSQC +/-	2	1	1	2	1	1	3	1	2	1	1	0	1	1	0
HSQC +	5	5	0	6	5	1	7	5	2	10	6	4	10	6	4

Results are shown for all proteins and separated for the 45 proteins ≤ 21 kDa and the 51 proteins > 21 kDa.

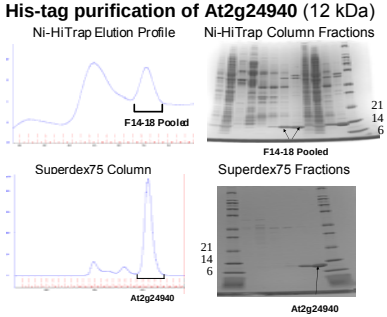
In general, the wheat germ cell-free platform offers the advantage of greater genome coverage for NMR-based structural proteomics, whereas the *E. coli* platform when successful yields more protein, as currently needed for crystallization trials for X-ray structure determination.

Improvement in the progress of targets carried through CESG pipeline protocols

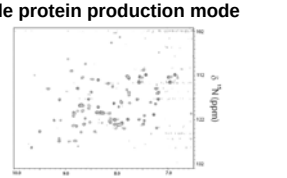
(Columns 2-7) Improvement in the progress of the 96 targets carried through each of the three protein production protocols by following up with a second protocol. **(Column 8)** Improvement achieved over the cell-based results by utilizing the two cell-free protocols. (**Column 9**) Improvement achieved over the cell-free methods by carrying the targets through the cell-based protocol.



His-tag purification of At2g24940 (12 kDa)



Spectrum of a ^{15}N-protein prepared in small-scale protein production mode



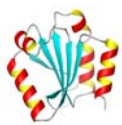
At2g24940 (13.5 KDa) was synthesized (~80-100 µg) in the small-scale protein production mode on the GeneDecoder1000™. Sixteen wells were devoted to this protein with ~5 µg/well average yield. The spectrum was collected on a 500 MHz Bruker spectrometer equipped with a cryogenic probe (160 transients with 64 complex points in the indirect (^{15}N) dimension; total acquisition time 6 h).

Gene Decoder1000™	Protemist10™
Screening Mode	**Large-Scale Protein Production Mode**
4 x 96-well plates:	*8 x 4 ml reactions:*
• Overnight run • 2-5 µg protein / well • Uses 2.5 – 5 mL of wheat germ extract / plate	• Overnight run • 1 – 3 mg protein / reaction • Uses 3 ml of wheat germ extract / reaction
Small-Scale Protein Production Mode	
2 x 96-well plates:	
• Overnight run • 10-20 µg protein / well • Uses 5 – 10 mL of wheat germ extract / plate	

Wheat germ cell-free structure gallery

At3g01050.1
12 kDa

At2g24940.1
14 kDa



At3g51030.1
13 kDa

At5g66040.1
14 kDa

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