Cell-Free with Increased Solubility

Expression of Soluble Native Human Proteins in Cell-Free Extracts

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Abstract

This article compares soluble native protein expression from Wheat Germ Extract Plus and two E. coli *expression systems using Flexi® Vector plasmids encoding 55 different human proteins. While only three of the proteins were expressed in the soluble fraction of* E. coli *cells, the number of proteins expressed using the* E. coli *T7 S30 Extract System for Circular DNA increased to 42. However, only 10 of those proteins were in the soluble fraction. In contrast, all 55 proteins were expressed in the soluble fraction of Wheat Germ Extract Plus, and nearly fourfold higher yields were achieved using the Wheat Germ Extract Plus in dialysis mode compared to batch mode.*

While *E. coli* is the first choice due to cost considerations, the expression of mammalian proteins is often problematic, as the protein produced is insufficient in yield or solubility.

Introduction

Choosing an expression system for the production of a recombinant protein can be difficult because not every protein is expressed to desired levels or solubility in every system. While *E. coli* is the first choice due to cost considerations, the expression of mammalian proteins is often problematic, as the protein produced is insufficient in yield or solubility. Cell-free extracts offer an alternative means to produce protein, but in the past, yields were insufficient for some downstream applications. Wheat Germ Extract Plus (Cat.# L3250) provides a higher yield of soluble protein (10–80µg/ml), and the yield can be increased using dialysis methods (1). For this article, we screened different protein expression systems using the Flexi® Vector technology[a,b] to reduce the cloning burden. The Flexi® Vector Systems provide an easy way to transfer the protein-coding region from one vector type to another in a high-fidelity manner, where each vector is optimal for expressing recombinant protein in a particular system (2).

We compared the expression of 55 human proteins in *E. coli* BL21(DE3)pLysS Competent Cells (Cat.# L1191) to expression in the *E. coli* T7 S30 Extract System for Circular DNA (Cat.# L1130) and in Wheat Germ Extract Plus. These proteins are all encoded by their native sequences (i.e., codons have not been optimized for the expression system), and all lack affinity or solubility tags. We also used Wheat Germ Extract Plus to examine the functional activity of one of the expressed proteins that could be readily assayed (activated procaspase-3 with the Caspase-Glo® 3/7 Assay, Cat.# G8090) and showed nearly a fourfold increase of functional protein using a dialysis method.

Creating a Library of Human Recombinant Protein Clones

Flexi® Vector Systems employ a flexible, directional cloning method to produce plasmids that express protein-coding regions with or without peptide fusion tags. The features necessary for expression and the options for protein fusion tags are carried on the vector backbone, and the protein-coding region can be shuttled between vectors using two rare-cutting restriction endonucleases, *Sgf* I[c] and *Pme* I[d]. The Flexi® Vectors contain a lethal gene, barnase, for positive selection of the protein-coding sequence and an antibiotic resistance marker for selection of colonies containing the Flexi® Vector. These systems provide a rapid, efficient and high-fidelity way to transfer sequences between a variety of Flexi® Vectors.

We obtained recombinant human protein clones from several sources: Origene (MD, USA), Kazusa DNA Research Center (Chiba, Japan), the IMAGE consortium and MGC (via ATCC, VA, USA). The coding regions obtained from Origene and Kazusa were in Flexi® Vector format, flanked by *Sgf* I and *Pme* I sites. cDNA clones from the IMAGE and MGC collections were converted into Flexi® Vector format by amplifying the protein-coding region with primers that append *Sgf* I and *Pme* I sites to the coding region (*3*).

For the *E. coli* experiments, protein-coding regions were either already in or were transferred into pF1A or pF1K T7 Flexi® Vector (Figure 1, Panel A). These vectors direct transcription of protein-coding regions via the T7 RNA polymerase promoter, and in vivo protein expression using these vectors requires use of a bacterial strain expressing T7 RNA polymerase, like the BL21(DE3)pLysS Cells. For the wheat germ experiments, protein-coding regions were transferred into pF3A or pF3K WG (BYDV) Flexi® Vector (Figure 1, Panel B). These vectors are designed for in vitro protein expression in wheat germ extracts via the T7 or SP6 RNA polymerase promoters. They also contain 5´ and 3´ translation-enhancing sequences of Barley Yellow Dwarf Virus (BYDV), which can increase protein expression in wheat germ extracts (4,5).

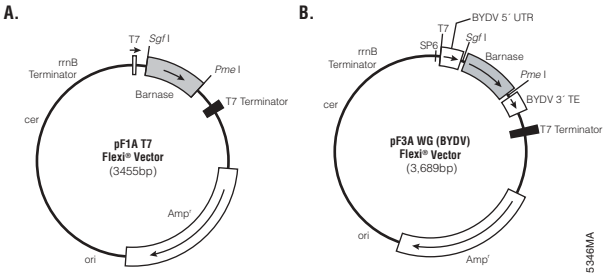

Figure 1. Flexi® Vectors used to express human proteins. Panel A. The pF1A T7 Vector. **Panel B.** The pF3A WG (BYDV) Vector. Plasmids are also available with markers for kanamycin or ampicillin resistance.

For the following experiments, plasmid DNA from these clones was purified using the PureYield™ Plasmid Midiprep System (Cat.# A2492). For the in vivo *E. coli* experiments, the pF1A or pF1K plasmid DNA was transformed into BL21(DE3)pLysS Competent Cells. Table 1 lists the proteins assembled in this library, with their accession numbers and expected protein molecular weight. The library includes representatives from kinase, transcription factor, receptor and protease families. Human protein-coding regions listed in Table 1 replace the shaded sequence labeled Barnase (Figure 1) in this study of heterologous protein expression.

Expression of Human Proteins in *E. coli* BL21(DE3)pLysS

E. coli BL21(DE3)pLysS cells contain T7 RNA polymerase under the control of an IPTG-inducible promoter. All 55 human protein clones were transformed into this strain and examined for the ability to produce protein of the expected size on Coomassie®-stained gels. Only three of the 55 human clones produced soluble protein as detected by SDS-PAGE analysis; one of those clones is shown in Figure 2. While this frequency of expression is a bit low, it is not far from the expected expression frequency when considering that our assembled library contains a large number of kinases and other proteins known to be difficult to express in *E. coli*. Factors that influence protein expression in *E. coli* include codon usage, fusion to solubility-enhancing peptides, message stability, chaperone-assisted folding, cellular toxicity, post-translational modification and the presence of a protein binding partner, to name a few. For comparison, protein expression was also evaluated in the cell-free *E. coli* T7 S30 Extract System for Circular DNA and Wheat Germ Extract Plus.

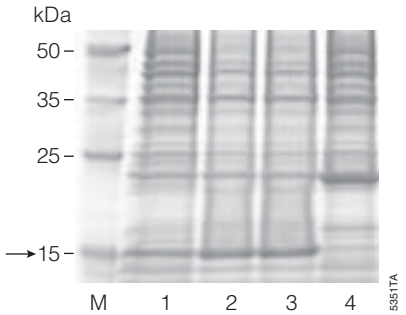

Figure 2. In vivo *E. coli* expression of sample 49 in BL21(DE3)pLysS Competent Cells (Cat.# L1191). Cultures in LB at 37°C were induced with 1mM IPTG when they reached an $O.D._{600}$ of 0.5. Induction proceeded for 4.5 hours at 37°C, after which the cells were pelleted and frozen overnight at –20°C. Cells were resuspended in 20mM HEPES (pH7.2), 2mM EDTA with 50µg/µl of lysozyme and completely lysed by sonication (Branson Model 2510, 3 times for 5 minutes each). Samples were separated on 4–20% Tris-Glycine gels (Invitrogen Cat.# EC6025) Analysis was done on Amersham Personal Densitometer SI. Lane M: Promega Broad Range Protein Molecular Weight Markers (Cat.# V8490); lane 1: Uninduced whole cell lysate; lane 2: Induced whole cell lysate; lane 3: Induced supernatant; and lane 4: Control BL21(DE3)pLysS cells. For the induced supernatant, lane 3, the lysates were centrifuged at 16,000 × *g* for 15 minutes at 22°C. The arrow indicates the expected size of the protein, 15,747Da.

Expression of Human Proteins in *E. coli* T7 S30 Extract System

The *E. coli* T7 S30 Extract System for Circular DNA contains T7 RNA polymerase as well as all the components required for transcription and translation, simplifying expression of DNA sequences cloned into pF1A or pF1K T7 Flexi® Vectors (Cat.# C8441, C8451), which contain a T7 RNA polymerase promoter. After addition of template DNA, protein expression was carried out for 2 hours at 37°C and monitored non-radioactively using the FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System (Cat.# L5001). Solubility of the expressed protein was evaluated by comparing the labeled protein band on SDS-PAGE before and after centrifugation (Figure 3, Panel A). Forty-two of the 55 human proteins examined (76%) were expressed, but only 10 (24% of those expressed) were at least 50% soluble (Table 1).

Expression of Human Proteins in Wheat Germ Extract Plus

Wheat Germ Extract Plus is an optimized extract that contains all the necessary components for efficient translation of a wide range of mRNA templates (1). For the following experiments, transcripts were generated from pF3A or pF3K circular plasmid templates using T7 RiboMAX™ Express Large Scale RNA Production System (Cat.# P1320) followed by gel filtration clean-up using MicroSpin® G-25 Columns (Amersham). Approximately 8µg of mRNA was used to program the translation reaction in Wheat Germ Extract Plus. Protein expression was monitored non-radioactively using

Table 1. Summary of Protein Library Used in this Study. Samples 1–40 were obtained from Origene (MD, USA), and samples 41–44 were from Kazusa DNA Research Center. Samples 1–44 were provided in Flexi® Vectors. Samples 45–55 were directed into Flexi® Vectors by PCR cloning (3), and the source templates were cDNA clones from ATCC (VA, USA), either from the IMAGE or MGC collections. BL21 = *E. coli* BL21(DE3)pLysS Competent Cells. S30 = *E. coli* T7 S30 Extract System for Circular DNA. WG = Wheat Germ Extract Plus. ACCN = Accession number. N = Not expressed. I = Expressed but insoluble. S = Expressed and soluble. Solubility is defined as greater than 50% of expressed protein remaining in the soluble fraction after centrifugation at 16,000 × *g* for 15 minutes at 22°C.

Sample	Description	ACCN	Mol. Wt.	BL21	S30	WG
1	Androgen receptor (dihydrotestosterone receptor; AR)	NM_000044	99287	N	I	S
2	Estrogen receptor 1 (ESR 1)	NM_000125	66315	N	N	S
3	5-hydroxytrptamine (serotonin) receptor 1A (HTR1A)	NM_000524	46206	N	I	S
4	Glutamate receptor, metabotropic 2 (GRM2)	NM_000839	95667	N	N	S
5	Glutamate receptor, metabotropic 3 (GRM3)	NM_000840	98979	N	I	S
6	Mitogen-activated protein kinase 14 (MAPK14)	NM_001315	41393	N	S	S
7	Heat shock factor binding protein 1 (HSBP1)	NM_001537	8643	N	N	S
8	B-cell translocation gene 1 anti-proliferative (BTG1)	NM_001731	19308	N	I	S
9	fms-related tyrosine kinase (FLT1)	NM_002019	150834	N	N	S
10	Integrin, alpha V, vitronectin receptor (ITGAV)	NM_002210	116151	N	I	S
11	Kinase insert domanin receptor (a type III receptor tyrosine kinase; KDR)	NM_002253	151640	N	I	S
12	Protein kinase C, gamma (PRKCG)	NM_002739	78547	N	N	S
13	Mitogen-activated protein kinase 9 (MAPK9)	NM_002752	48238	N	S	S
14	Mitogen-activated protein kinase kinase 6 (MAP2K6)	NM_002758	37592	N	I	S
15	Mitogen-activated protein kinase kinase 4 (MAP2K4)	NM_003010	44387	N	I	S
16	Aurora kinase C (AURKC)	NM_003160	32287	N	I	S
17	Aurora kinase B (AURKB)	NM_004217	39380	N	I	S
18	cAMP responsive element binding protein 1 (CREB1)	NM_004379	35236	N	S	S
19	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2)	NM_004448	138026	N	N	S
20	MCM2 minichromosome maintenance deficient 2 mitotin (MCM2)	NM_004526	101996	N	I	S
21	MCM5 minichromosome maintenance deficient 5 cell division cycle 46 (MCM5)	NM_006739	82385	N	N	S
22	Calcium binding protein P22 (CHP)	NM_007236	22555	N	N	S
23	Nuclear receptor binding protein (NRBP)	NM_013392	59944	N	I	S
24	Mitogen-activated protein kinase 8 (MAPK8)	NM_139049	48395	N	I	S
25	MCM4 minichromosome maintenance deficient 4 (MCM4)	NM_182746	96657	N	I	S
26	Growth factor, augmenter of liver regeneration (GFER)	XM_034465	15495	N	S	S
27	Bruton agammaglobulinemia tyrosine kinase (BTK)	NM_000061	76381	N	I	S
28	Glutamate receptor, metabotropic 5 (GRM5)	NM_000842	128973	N	I	S
29	Spleen tyrosine kinase (SYK)	NM_003177	72066	N	I	S
30	tec protein tyrosine kinase (TEC)	NM_003215	34649	N	I	S
31	PTK2B protein tyrosine kinase 2 beta (PTK2B)	NM_004103	115935	N	I	S
32	EPH receptor B3 (EPHB3)	NM_004443	110429	N	I	S
33	Receptor tyrosine kinase-like orphan receptor 1 (ROR1)	NM_005012	104412	N	I	S
34	Colony stimulating factor 1 receptor (CSF1R)	NM_005211	108083	N	I	S
35	Discoidin domain receptor family, member 2 (DDR2)	NM_006182	96836	N	N	S
36	Cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4)	NM_017460	57542	N	S	S
37	Neurotrophic tyrosine kinase (NTRK3)	NM_002530	929015	N	N	S
38	fms-related tyrosine kinase 3 (FLT3)	NM_004119	112904	N	N	S
39	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT)	NM_000222	109964	N	I	S
40	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1 (PTPRZ1)	NM_002851	254630	N	I	S
41	Protein tyrosine phosphatase receptor type T(PTPRT)	AB006621	165434	S	I	S
42	DnaJ (Hsp40) homolog, subfamily c, member 6 (DNAJC6)	AB007942	102887	S	I	S
43	Myotubularin-related protein 3 (MTMR3)	AB002369	154450	N	N	S
44	TAO kinase 1 (TAOK1)	AB037782	116533	N	I	S
45	Histone deacetylase (HD1)	U50079	55202	N	I	S
46	Caspase-6, apoptosis-related cysteine protease	BC000305	33409	N	I	S
47	Cysteine protease Yama (procaspase-3)	U26943	31707	N	S	S
48	Mineralcorticoid receptor (hMR)	M16801	107167	N	S	S
49	Cellular retinol binding protein II (CRBPII)	U13831	15747	S	I	S
50	Glucocorticoid receptor (GRL)	NM_000176	85759	N	S	S
51	Procaspase-7, cysteine protease CHM-1	U40281	34376	N	I	S
52	Melanoma antigen recognized by T cells (MAR1)	BC014423	13256	N	N	S
53	Small muscular protein (SMPX)	BC005948	9658	N	S	S
54	T-cell activation protein (Q9Y605)	BC022797	14749	N	I	S
55	T-cell tumor protein (TCTP)	BC003352	19695	N	S	S

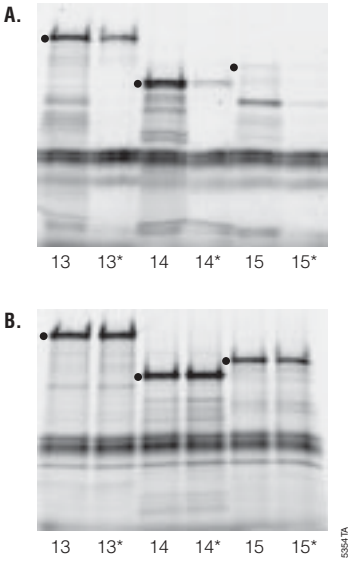

Figure 3. Solubility of proteins expressed in cell-free extracts. Solubility of the expressed protein was evaluated by comparing the labeled protein band on SDS-PAGE before and after centrifugation. The reactions were centrifuged at 14,000rpm at room temperature for 15 minutes, using an Eppendorf centrifuge 5415 C to prepare the soluble fraction. The 4–20% Tris-Glycine gels (Invitrogen, Cat.# EC6025) were loaded with 1µl of each reaction before and after centrifugation. The gels were analyzed by fluorometry using Amersham Typhoon® Blue2 (488) Laser for excitation and the 520BP 40CY2 Blue Fam emission filter. Samples 13, 14 and 15 after centrifugation are indicated as samples 13*, 14* and 15*. **Panel A.** The cell-free protein expression was performed using the *E. coli* T7 S30 Extract System for Circular DNA (Cat.# L1130) with approximately 4µg of circular DNA, 1µl of FluoroTect™ $Green_{Lys}$ tRNA (Cat.# L5001), 2µl of Amino Acid Mixture Minus Methionine and 2µl of Amino Acid Mixture Minus Leucine, using the protocol found in Technical Bulletin #TB219. The reactions were incubated at 37°C for 2 hours. **Panel B.** Translations were performed in Wheat Germ Extract Plus using approximately 8µg of mRNA and 1µl of FluoroTect™ $Green_{Lys}$ tRNA for 2 hours at 25°C. Dots indicate the expected protein size of each sample.

FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System (Cat.# L5001; Figure 4). Solubility of the expressed protein was evaluated by comparing the labeled protein band on SDS-PAGE before and after centrifugation. All 55 human proteins examined were expressed with greater than 50% solubility (Figure 3, Panel B; Figure 4).

Increased Protein Yield Using Dialysis in Wheat Germ Extract Plus

Wheat Germ Extract Plus is typically used in a single-tube format (batch mode) to express proteins in the range of 10–80µg/ml (1). This yield can be increased by dialysis. We examined the expression of eight of the human proteins in dialysis mode. Based on Coomassie®-stained SDS-PAGE, yield for these proteins is estimated at 250µg/ml. One of the samples tested for functional activity was procaspase-3, sample 47. After activating the procaspase-3 enzyme with caspase-8, we assayed activity using the Caspase-Glo® 3/7 Assay (Cat.# G8090). Protein expressed in dialysis mode for 7 hours provided a nearly fourfold increase in caspase-3 activity compaired to a single-tube format. Assuming equivalent activities of a recombinant caspase-3 standard (BioMol) and the proenzyme produced in wheat germ and processed by caspase-8, batch mode produced 34µg/ml caspase-3, and dialysis mode produced 120µg/ml. This is consistent with our previous results of a four- to sevenfold increase in protein production by dialysis mode observed with reporter proteins (reference 1 and data not shown).

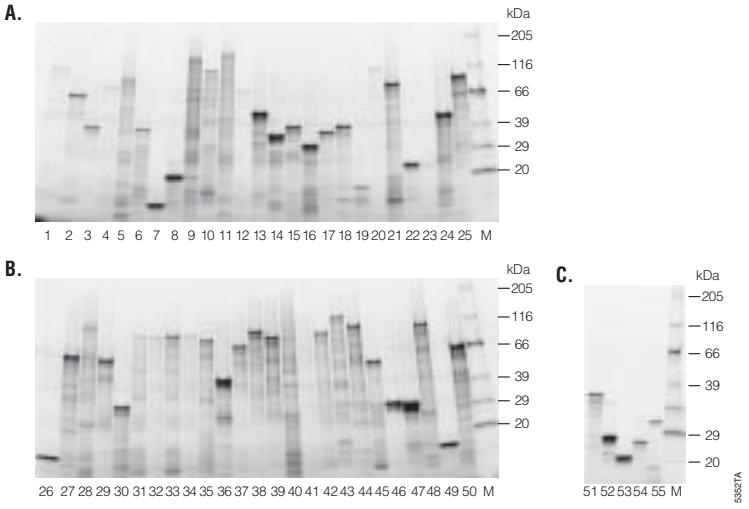

Figure 4. Wheat Germ Extract Plus expression of 55 human proteins. Flexi® Vector plasmid DNA was purified using the combination protocol of the PureYield™ Plasmid Midiprep System (Cat.# A2492). Transcripts were generated from 55 circular plasmid templates using T7 RiboMAX™ Express Large Scale RNA Production System (Cat.# P1320) followed by gel filtration clean-up (MicroSpin® G-25 Columns, Amersham Cat.# 27-5325-01). Translations were performed in Wheat Germ Extract Plus (Cat.# L3250) using approximately 8µg of mRNA and 1µl of FluoroTect™ $Green_{Lys}$ tRNA (Cat.#. L5001) at 25°C for 2 hours. After the translations were completed, 5µl of each reaction was incubated with 1µl of RNase ONE™ Ribonuclease (Cat.# M4261) and 4µl of water for 15 minutes at room temperature to remove unincorporated FluoroTect™ lysine-charged tRNA. Two microliters of RNase ONE™-Ribonuclease treated samples were mixed with 10µl of 4X SDS sample buffer and heated for 1 minute at 65°C and seperated on 4–20% Criterion™ Precast gels (Bio-Rad Cat.# 345-0034) at 150 volts for 1.5 hours. Lane M: fluorescence molecular weight standards (Sigma, Cat.# F3526) were diluted in 4X SDS sample buffer 1:100 before loading 1µl per lane. Gels were analyzed by fluorometry using Amersham's Typhoon® Blue2 (488) Laser for excitation and 520BP 40CY2 Blue Fam emission filter.

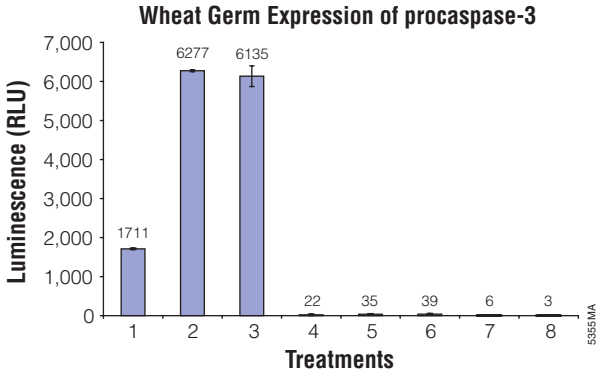

Figure 5. Wheat Germ Extract Plus expression of procaspase-3. Batch mode reactions (treatments 1 and 4) were performed with 8µg of mRNA and 30µl of Wheat Germ Extract Plus in 50µl reactions for 2 hours at 25°C. Treatments 7 and 8 are batch mode controls that lack input RNA. Dialysis mode reactions (treatments 2, 3, 5 and 6) were performed with 12µg of mRNA and 30µl of Wheat Germ Extract Plus in 50µl reactions for either 7 (treatments 2 and 5) or 18 hours (treatments 3 and 6) at 25°C, in dialysis cups (MWCO 12,000 BioTec International, sold by Daiichi Pure Chemicals DBC Code 212956) and 2.5ml of dialysis buffer in a Uniplate (Watman Cat.# 7701-5102), The dialysis buffer consisted of 12mM HEPES, 0.5mM spermidine, 5mM DTT, 80µM amino acids, 100mM KOAc, 1.2mM ATP, 0.1mM GTP, 10mM CP and 1.5mM $Mg(OAc)_2$.

Caspase 3 activity was measured wth Caspase-Glo® 3/7 Assay (Cat.# G8091). Procaspase-3 was activated by treating 20µl of lysate with 250U caspase-8 (2.5µl BioMol caspase-8) for 1 hour at 37°C (treatments 1, 2, 3 and 7). Nontreated, matched lysates (treatments 4, 5, 6 and 8) recieved 2.5µl of 10mM HEPES (pH 7.5) and were incubated for 1 hour at 37°C. After treatment or mock treatment with caspase 8, lysates were diluted to a final dilution of 1:20,000 in 10mM HEPES (pH 7.5). Caspase-Glo® 3/7 Assay measurements were made at steady state (approximately 20 minutes). Results are presented as relative luminescent unis (RLU). A standard curve (r^2 = 0.9994) was generated using recombinant human caspase-3 (BioMol SE-169, diluted 1:20,000 from 200U/ml in Wheat Germ Extract Plus lacking input RNA), whose specific activity was 13,889 Units/µg (BioMol defines one activity unit as producing 1pmol/minute of pNA product using Ac-DEVD-pNA at 200µM at 30°C).

Summary

We compared soluble native protein expression from Wheat Germ Extract Plus and two *E. coli* expression systems using Flexi® Vector plasmids encoding 55 different human proteins. While only three of the proteins expressed in the soluble fraction of *E. coli* BL21(DE3)pLysS Cells, the number of proteins expressed in *E. coli* T7 S30 Extract System for Circular DNA increased to 42, although only 10 of those proteins were in the soluble fraction. All 55 proteins were expressed in the soluble fraction of Wheat Germ Extract Plus. We also demonstrated the production of the proenzyme form of caspase-3, which can be activated by caspase-8. Nearly fourfold higher yields were achieved using the Wheat Germ Extract Plus in dialysis mode compared to single-tube batch mode.

Acknowledgment

We would like to thank Dr. Vadim Smirnov for cloning and sequencing the IMAGE and MGC protein-coding regions in Flexi® Vectors.

References

1. Hurst, R. *et al.* (2005) *Promega Notes* **90**, 19–21.
2. Slater, M. *et al.* (2005) *Promega Notes* **89**, 11–5.
3. *Flexi® Vector Systems Technical Manual,* #TM254, Promega Corporation.
4. Guo, L., Allen, E.M. and Miller, W.A. (2000) *RNA* **6**, 1808–20.
5. Guo, L., Allen, E.M. and Miller, W.A. (2001) *Mol. Cell* **7**, 1103–9.

Protocols

- *Wheat Germ Extract Plus System Technical Manual* #TM066, Promega Corporation.
  www.promega.com/tbs/tm066/tm066.html
- *Flexi® Vector Systems Technical Manual* #TM254, Promega Corporation.
  www.promega.com/tbs/tm254/tm254.html
- *PureYield™ Plasmid Midiprep System Technical Manual* #TM253, Promega Corporation.
  www.promega.com/tbs/tm253/tm253.html

Ordering Information

Product	Size	Cat.#
Wheat Germ Extract Plus	40 × 50µl	L3250
	10 × 50µl	L3251
pF3A WG (BYDV) Flexi® Vector	20µg	L5671
pF3K WG (BYDV) Flexi® Vector	20µg	L5681
pF1A T7 Flexi® Vector	20µg	C8441
pF1K T7 Flexi® Vector	20µg	C8451

Related Products

Product	Size	Cat.#
BL21(DE3)pLysS Competent Cells	1ml	L1191
Caspase-Glo® 3/7 Assay*	2.5ml	G8090
E. coli T7 S30 Extract System for Circular DNA	30 reactions	L1130
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System*	40 reactions	L5001
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495

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