^{15}N Labeling of Proteins Overexpressed in the *Escherichia coli* Strain KRX

ABSTRACT We describe a protocol for efficient ^{15}N-labeled protein production in the *E. coli* strain, KRX. This procedure can help streamline the process of target cloning, protein expression, screening and preliminary protein folding/aggregation assessment by NMR spectroscopy and other methods that require ^{15}N protein labeling.

Kate Qin Zhao, Jim Hartnett, and Michael R. Slater, Promega Corporation

INTRODUCTION

High-efficiency cloning and tightly regulated recombinant protein production in *E. coli* is achieved in the highly competent Single Step (KRX) Competent Cells[a] (1). In this strain, the T7 RNA polymerase is controlled by the rhaP$_{BAD}$ promoter, which is posi-tively activated by rhamnose and catabolically repressed by glucose (1).

Previously, we demonstrated efficient selenomethionine (Se-Met) incorporation in KRX (2). Here we describe a protocol for efficient ^{15}N-labeled protein production of Monster Green® GFP and human cellular retinol-binding protein type II (CRBPII, GenBank® accession # U13831) using the pFN6A (HQ) Flexi® Vector (Cat.# C8511). The expressed proteins were purified with the HisLink™ Resin (Cat.# V8821) using the N-terminal HQ tag.

Table 1. Protocol for ^{15}N Labeling of Protein Using the Single Step (KRX) Competent Cells.

Day 1	
1.	Inoculate a single colony from freshly streaked plate into 5 ml of starter culture: LB broth with 0.4% glucose and 100 mg/L ampicillin.
2.	Grow at 37 °C for ~6–8 hours.
3.	Inoculate the starter culture into 250 ml of overnight culture: 250 ml of LB broth + 0.2% glucose with 100 mg/l ampicillin.
4.	Grow cells overnight at 37 °C (<18 hours).
Day 2	
5.	Harvest by centrifugation at 3,000 rpm (Beckman JA 14 rotor) for 10 minutes at 4 °C.
6.	Resuspend cell pellet in 250 ml (volume equal to overnight culture) of induction media: 1X M9 media (7) with the carbon source (glucose) substituted by 1% (v/v) glycerol, supplemented with 1X metal mix (8), with 1 g/L of NH_4Cl or $^{15}NH_4Cl$ (Cambridge Isotopes).
7.	Adapt the cells at 37 °C for 30 minutes to 1 hour.
8.	Induce with 0.2% (w/v) rhamnose and 0.4 mM cAMP overnight at 25 °C.
Day 3	
9.	Collect cells by centrifugation at 5,000 rpm (Beckman JA 14 rotor) for 20 minutes at 4°C.
10.	Resuspend cell pellet in 25 ml of 1X FastBreak™ Cell Lysis Reagent (Cat.# V8571) with 25 µl of RQ1 RNase-Free DNase (Cat.# M6101) and protease inhibitor cocktail (Roche) in 50 ml
11.	Incubate cell resuspension at room temperature for 20 minutes.
12.	Collect the supernatant by centrifugation at 7,500 rpm (Beckman JA 14 rotor) for 20 minutes at 4°C.
13.	Load the supernatant by gravity onto a pre-equilibrated 1 ml HisLink™ Protein Purification Resin (Cat.# V8821), wash with 20 ml (20X column volume) of binding/wash buffer: 10 mM HEPES (pH 7.5), 500 mM NaCl and 10 mM imidazole.
14.	Elute with 10 ml (10X column volume) of elution buffer: 10 mM HEPES (pH 7.5), 500 mM NaCl and 500 mM imidazole.

^{15}N PROTEIN LABELING

For small proteins (≤20 kDa), the protein folding/aggregation state can be assessed rapidly by NMR spectroscopy, especially by 2D ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectroscopy (3,4 and references therein). 2D ^{1}H-^{15}N HSQC is also valuable for detecting protein:protein and protein:ligand interactions. An example is SAR (structure activity relationships) by NMR (5).

For recent mass spectroscopy applications, labeling with stable isotopes such as ^{15}N can add signature mass tags to peptides or proteins for quantification and source identification (6 and references therein). We developed a protocol (Table 1) for efficient ^{15}N-labeled protein production in KRX cells.

Protein yield was estimated by absorbance at A_{280}. The molecular weights of purified proteins were determined by MALDI TOF mass spectroscopy (HT Laboratories, Inc.). Labeling efficiency was calculated as described in reference 2.

The results of ^{15}N labeling are summarized in Table 2. For the two test proteins, we averaged ~97% ^{15}N incorporation. The overlay of MALDI TOF traces for CRBPII (Figure 1) shows that we achieved uniform labeling with no mass peaks corresponding to the unlabeled protein in the ^{15}N sample. This further demonstrates the tighter control of basal-level protein production in KRX cells.

PROTEIN ANALYSIS BY ^{1}H-^{15}N HSQC

In a ^{1}H-^{15}N HSQC spectrum, each cross peak represents a unique proton attached to a ^{15}N atom, such as a protein backbone amide, side chain amide (Asn, Gln) and imino (Trp) protons. A well folded,

Table 2. ^{15}N Incorporation Efficiency.

		Unlabeled (Da)	^{15}N (Da)	% Labeling
Monster Green® GFP		27,346	27,655	94.8
CRBPII	1	17,012	17,231	97.8
	2	17,017	17,224	>99

The molecular weights of purified proteins were determined by MALDI TOF mass spectrometry (HT Laboratories, Inc). CRBPII proteins were prepared and evaluated from two separate cell cultures. Theoretical size of unlabeled Monster Green® GFP is 27,349 Da and of CRBPII is 16,958 Da; molecular weight of ^{15}N fully substituted Monster Green® GFP is 27,675 Da and of CRBPII is 17,153 Da as calculated by Protein Calculators v3.3 (**w.scripps.edu/~cdputnam/protcalc.html**).

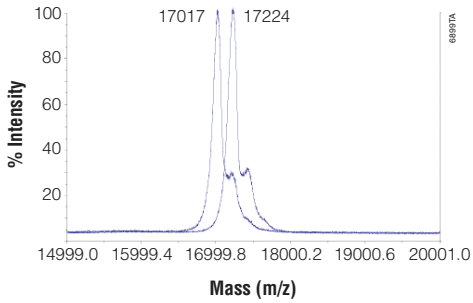

Figure 1. Overlay of MALDI TOF mass spectrometry traces of the unlabeled and ^{15}N-labeled protein. The protein concentration was ~10 mg/ml; samples were analyzed by HT Laboratories, Inc. (San Diego, CA).

nonaggregating protein should have a well dispersed cross peak pattern in the ^{1}H-^{15}N HSQC spectrum. The total number of cross peaks in the spectrum should be close to the sum of the protons described above for NMR spectroscopy to successfully determine structure.

We produced and purified to near homogeneity ~30–35 mg/L of CRBPII from KRX cells (Figure 2, Panel A). The ^{1}H-^{15}N HSQC data generated with this protein showed that it is folded (Figure 2, Panel B). There is a well dispersed peak pattern, and around 161 out of the possible 183 cross peaks are present (near 90% coverage), including all those for the Asn, Gln and Trp side chains and the N-terminal HQ tag.

CONCLUSIONS

We have demonstrated that the KRX strain is suitable for applications that require efficient ^{15}N incorporation into target proteins. These cells can be used for target cloning, protein expression, screening and protein folding/aggregation assessment by ^{15}N HSQC (3–5) or protein identification/quantitation by mass spectrometry (6). Single Step (KRX) Competent Cells eliminate the need to transfer the expression plasmid into another expression/labeling strain.

REFERENCES

1. Hartnett, J., Gracyalny, J. and Slater M.R. (2006) *Promega Notes* **94**, 27–30.

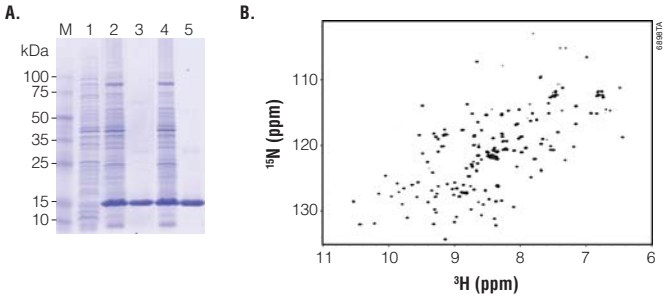

Figure 2. ^{15}N- labeled CRBPII expression. Panel A. Proteins from uninduced cells, induced cell lysate supernatant and eluted from HisLink™ Resin were analyzed by SDS-PAGE on 4–20% Tris-Glycine gels followed by Coomassie® blue staining. Lanes BI and S, protein equivalent to 15 µl of cell culture; lanes E, protein equivalent to 40 µl of cell culture; lane M, Broad Range Protein Molecular Weight Markers (Cat.# V8491). **Panel B.** ^{1}H-^{15}N HSQC spectrum of ~1 mM ^{15}N CRBPII in 10 mM phosphate buffer (pH 6.8) with 130 mM NaCl at 25 °C. The data was recorded at the National Magnetic Resonance Facility (Madison, WI; NMRFAM) on a 750 mHz Bruker spectrometer equipped with a cryogenic ^{1}H, ^{15}N, ^{13}C triple-resonance probe, with 1,024 and 200 complex data points for proton and nitrogen, respectively. Four scans were accumulated for each increment. Total data-collection time was 27 minutes.

2. Zhao, K.Q., Hartnett, J. and Slater, M.R. (2007) *Promega Notes* **96**, 24–6.
3. Yee, A., Gutmanas, A. and Arrowsmith, C.H. (2006) *Curr. Opin. Struct. Biol.* **16**, 611–7.
4. Yee, A.A. *et al.* (2005) *J. Am. Chem. Soc.* **127**, 16512–7.
5. Shuker, S.B. *et al.* (1996) *Science* **274**, 1531–4.
6. Pan, S. and Aebersold, R. (2006) *Methods Mol.Biol.* **367**, 209–18.
7. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
8. Studier, F.W. (2005) *Protein Expr. Purif.* **41**, 207–34.

ORDERING INFORMATION

Product	Size	Cat.#
Single Step (KRX) Competent Cells	5 × 200 µl	L3001
	20 × 50 µl	L3002

[a] **Usage Restrictions for the T7 Expression System**

The T7 expression system is based on technology developed at Brookhaven National Laboratory under contract with the U.S. Department of Energy and is the subject of patents assigned to Brookhaven Science Associates, LLC (BSA). This technology, including bacteria, phage and plasmids that carry the gene for T7 RNA polymerase, is to be used for academic or nonprofit laboratory or licensed commercial research purposes only. By accepting or using the T7 expression technology you agree to be bound by the following conditions set forth by BSA. The initial purchaser may refuse to accept the conditions of this notice by returning this product and the enclosed materials to Promega unused.

Academic and NonProfit Laboratories

No materials that contain the cloned gene for T7 RNA polymerase may be distributed further to third parties outside of your laboratory unless the recipient receives a copy of this assurance notice and agrees to be bound by its terms. This limitation applies to Bacterial Strains JM109(DE3), BL21(DE3)pLysS and KRX and to any derivatives thereof.

Commercial Laboratories

A license is required for any commercial use of the T7 expression system, including use of the T7 system for research purposes or for production purposes by any commercial entity. Information about commercial licenses may be obtained from the Licensing Office, Brookhaven National Laboratory, Upton, NY 11973, Telephone: 631-344-7134, FAX: 631-344-3729.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

meetings

FEBRUARY

Spurenworkshop 2008
February 15–16, 2008
Salzburg, Austria
www.r-km.de/spurenworkshop2008

American Academy of Forensic Sciences
February 18–23, 2008
Washington, DC, USA
www.aafs.org

Screening Europe/ADMET Europe 2008
February 19–20, 2008
Stockholm, Sweden
www.selectbiosciences.com

MARCH

United States and Canadian Academy of Pathology
March 3–5, 2008
Denver, CO, USA
www.uscap.org

Society of Toxicology
March 17–19, 2008
Seattle, WA, USA
www.eshow2000.com/toxexpo

Japan Society for Bioscience, Biotechnology, and Agrochemistry
March 27–28, 2008
Nagoya, Japan
www.jsbba.or.jp

APRIL

Society for Biomolecular Screening
April 6–10, 2008
St. Louis, MO, USA
www.sbsonline.org

American Association of Cancer Research
April 12–16, 2008
San Diego, CA, USA
www.aacr.org

Quantitative PCR
April 21–22, 2008
San Diego, CA, USA
www.healthtech.com

Mid-Atlantic Association of Forensic Scientists
April 28–May 2, 2008
Huntington, WV, USA
www.maafs.org

4th Annual Protein Engineering Summit
April 28–May 2, 2008
Boston, MA, USA
www.pegssummit.com

MAY

California Association of Criminalists Spring Meeting
May 5–9, 2008
San Diego, CA, USA
www.cacnews.org

Erratum

The X axis of Figure 2 in "^{15}N Labeling of Protein Overexpressed in the *Escherichia coli* strain KRX" Promega Notes 97, pages 28–9 was mislabeled. Below is the corrected figure with the legend.

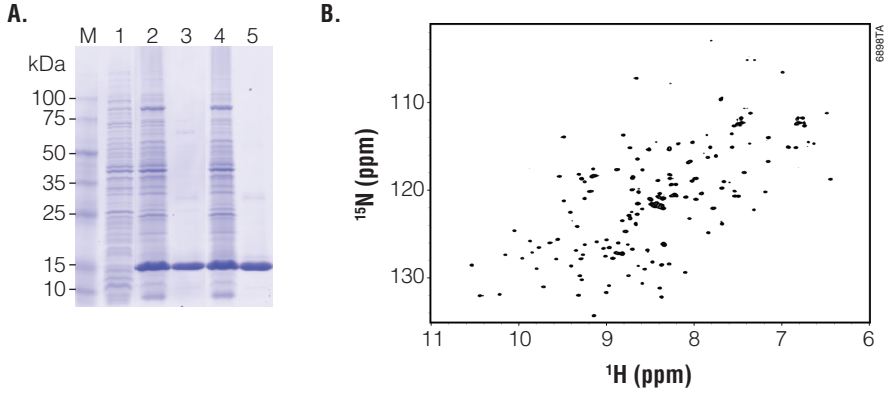

Figure 2. ^{15}N-labeled CRBPII expression. Panel A. Proteins from uninduced cells, induced cell lysate supernatant and eluted from HisLink™ Resin were analyzed by SDS-PAGE on 4–20% Tris-Glycine gels followed by Coomassie® blue staining. Lanes BI and S, protein equivalent to 15 µl of cell culture; lanes E, protein equivalent to 40 µl of cell culture; lane M, Broad Range Protein Molecular Weight Markers (Cat.# V8491). **Panel B.** ^{1}H-^{15}N HSQC spectrum of ~1 mM ^{15}N CRBPII in 10 mM phosphate buffer (pH 6.8) with 130 mM NaCl at 25 °C. The data were recorded at the National Magnetic Resonance Facility (Madison, WI; NMRFAM) on a 750 mHz Bruker spectrometer equipped with a cryogenic ^{1}H, ^{15}N, ^{13}C triple-resonance probe, with 1,024 and 200 complex data points for proton and nitrogen, respectively. Four scans were accumulated for each increment. Total data collection time was 27 minutes.