[U-^{13}C, ^{15}N] Protein Labeling Using *Escherichia coli* Strain KRX

ABSTRACT We have developed a protocol for efficient [U-^{13}C, ^{15}N]-labeled protein production in the highly competent *E. coli* strain, KRX, using Silantes CN OD2 medium. This procedure can facilitate protein analysis by NMR spectroscopy and other methodologies that require ^{13}C and ^{15}N protein labeling.

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KRX cells grown in Silantes CN OD2 media produce efficiently labeled protein for mass spectrometry and NMR analysis.

INTRODUCTION

The Single Step (KRX) Competent Cells[a,b] (1) can be used for cloning open reading frames (ORFs), expressing proteins under the control of the T7 promoter with rhamnose inducible T7 RNA polymerase, efficient incorporation of selenomethionine (Se-Met; 2), and [U-^{15}N] labeling (3).

We describe here a protocol for efficient [U-^{13}C, ^{15}N] protein labeling with human cellular retinol-binding protein type II (CRBPII, GenBank® Accession# U13831) in the pFN6A (HQ) Flexi® Vector using Silantes CN OD2 media. The labeled protein can be purified by HisLink™ Protein Purification Resin (Cat.# V8821) with the N-terminal HQ tag. ^{13}C and ^{15}N incorporation was assessed for the purified proteins using MALDI TOF mass spectroscopy. We also collected NMR spectra of two-dimensional ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) and 2D ^{1}H-^{13}C HSQC to demonstrate efficient isotope incorporation for both ^{15}N and ^{13}C. Labeling efficiency was calculated as described previously (2).

Our experiments averaged ~93% incorporation of ^{13}C and ^{15}N into CRBPII. Uniform labeling was shown by the overlay of MALDI TOF traces for CRBPII (Figure 1) with no mass peaks correlating to the unlabeled protein in the [U-^{13}C, ^{15}N] sample. The high level of ^{13}C incorporation indicated that unlabeled carbon atoms from rhamnose were not incorporated into the protein, since it was engineered to lack the ability to catabolize rhamnose as a carbon source (1). This validated the function of rhamnose as a protein-expression inducer rather than a carbon source for the KRX strain and further demonstrated the tight control of basal-level protein production (1–3).

Table 1. Protocol for Protein Expression and ^{13}C, ^{15}N Labeling with Silantes CN OD2 Medium.

Step
Day 1
1. Use a single colony from a freshly streaked plate to inoculate a starter culture in 5 ml of either Silantes OD2 (unlabeled) or CN OD2 (^{13}C, ^{15}N)-labeled medium (Cambridge Isotope Laboratories, Inc., and Silantes GmbH) and 100 mg/L of ampicillin.
2. Grow starter culture at 37 °C overnight.
Day 2
3. Inoculate 250 ml of Silantes OD2 or CN OD2 medium and 100 mg/L ampicillin using the 5 ml of the corresponding starter culture.
4. Grow cells at 37 °C until $O.D._{600}$ reaches ~1.
5. Induce with 0.2% (w/v) rhamnose, and continue at 37 °C for another 6 hours.
6. Centrifuge cells at 5,000 rpm (Beckman JA-14 rotor) for 20 minutes at 4 °C.
7. Resuspend cell pellet in 25 ml (1/10 of the induction media) of 1X FastBreak™ Cell Lysis Reagent (Cat.# V8571) with 25 µl (1:1,000 dilution) of RQ1 RNase-Free DNase (Cat.# M6101) and protease inhibitor cocktail (Roche) in 50 ml Falcon™ tubes.
8. Incubate the resuspended cells at room temperature for 20 minutes.
9. Pellet the cells by centrifuging at 7,500 rpm (Beckman JA 14 rotor) for 20 minutes at 4 °C.
10. Transfer the supernatant onto a pre-equilibrated 1 ml (1/250 volume of induction culture) HisLink™ Resin gravity column.
11. Wash column with 20 ml (20X column volume) of binding/wash buffer (10 mM HEPES [pH 7.5], 500 mM NaCl, 10 mM imidazole).
12. Elute protein with 10 ml (10X column volume) of elution buffer (10 mM HEPES [pH 7.5], 500 mM NaCl, 500 mM imidazole).

^{13}C, ^{15}N LABELING WITH SILANTES CN OD2 MEDIUM

In the KRX strain, the T7 RNA polymerase is controlled by $rhaP_{BAD}$ promoter, which is positively activated by rhamnose and catabolically repressed by glucose. This limits the use of ^{13}C glucose as the sole carbon source for ^{13}C protein labeling in KRX cells. Although ^{13}C glycerol can be used in place of ^{13}C glucose, the cost of this compound is currently five times more expensive per liter of M9 medium (or greater) than if using ^{13}C glucose.

Silantes-labeled media are suitable for protein-stable isotope labeling and support better cell growth than M9 media (www.silantes.com). According to Silantes literature and our experience, *E. coli* cells grow much better in Silantes OD2 medium than in M9 minimum media containing glucose. Using the ^{13}C, ^{15}N double-labeled Silantes CN OD2 medium, we achieved efficient cell growth and sufficient isotope incorporation for CRBPII protein expression levels when compared to those with unlabeled Silantes OD2 medium (Figure 2, Panel A). The cost of labeling with ^{13}C, ^{15}N double-labeled Silantes CN OD2 medium is lower than using ^{13}C glycerol

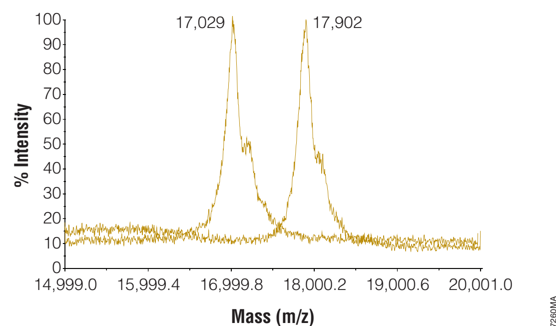

Figure 1. Overlay of MALDI TOF mass spectrometry traces of the unlabeled and ^{15}N-labeled protein. The protein samples were analyzed by HT Laboratories, Inc. (San Diego, CA). The protein concentrations used for this analysis were around 10 mg/ml, as estimated by A_{280} on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

and is slightly higher than using ^{13}C glucose-supplemented M9 medium. Therefore, Silantes medium is a good alternative for protein-isotope labeling when compared to the traditional M9 medium.

PROTEIN ANALYSIS BY NMR

We produced and purified to near homogeneity ~20 mg/L of CRBPII using KRX cells grown in Silantes CN OD2 medium (Figure 2, Panel A). In HSQC spectra, each peak represents a proton attached to a stable isotope-substituted heavy atom, such as ^{15}N or ^{13}C. The protein is likely to be folded, as revealed by ^{1}H-^{15}N HSQC analysis (Figure 2, Panel B). Two dimensional ^{1}H-^{13}C HSQC analysis verified ^{13}C incorporation (Figure 2, Panel C). We also successfully collected triple-resonance NMR data (not shown), which further validate sufficient ^{13}C and ^{15}N incorporation of the sample.

CONCLUSIONS

We demonstrated that our Single Step (KRX) Competent Cells are suitable for producing both ^{13}C and ^{15}N stable isotope-labeled proteins using Silantes CN OD2 medium. In addition, we show that rhamnose induced protein expression and is not used by KRX cells as a carbon source.

REFERENCES

1. Hartnett, J. *et al.* (2006) *Promega Notes* **94**, 27–30.
2. Zhao, K.Q. *et al.* (2007) *Promega Notes* **96**, 24–6.
3. Zhao, K.Q. *et al.* (2007) *Promega Notes* **97**, 28–9.

ORDERING INFORMATION

Product	Size	Cat.#
Single Step (KRX) Competent Cells	5 × 200 µl	L3001
	20 × 50 µl	L3002
L-Rhamnose Monohydrate	10 g	L5701
	50 g	L5702

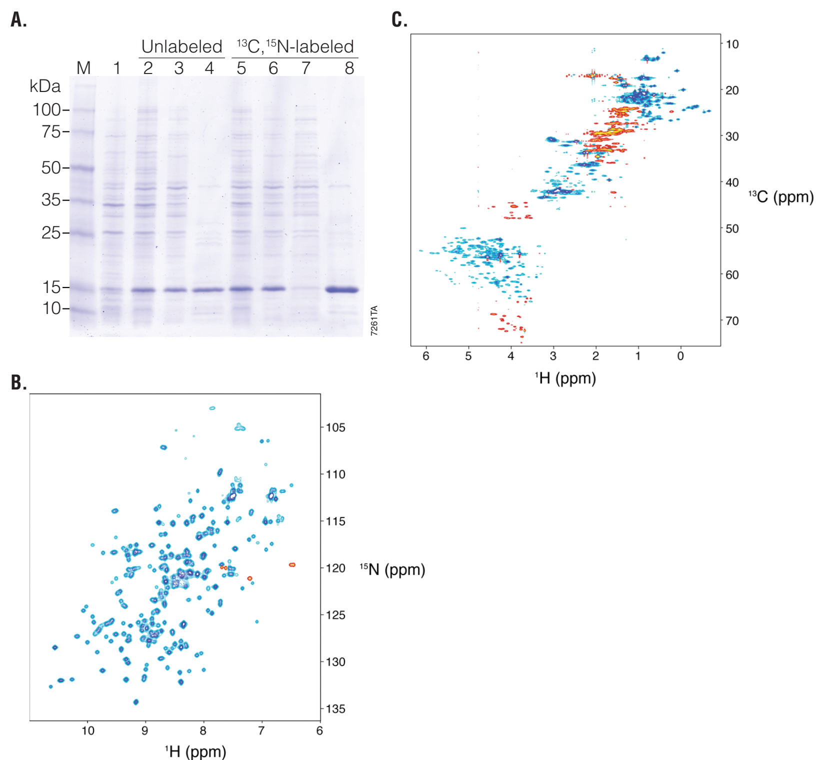

Figure 2. Expressing [*U*-^{13}C, ^{15}N]-labeled CRBPII. Panel A. Coomassie® blue-stained SDS-PAGE of labeled and unlabeled CRBPII. Unlabeled samples were grown in Silantes unlabeled OD2 medium; while [*U*-^{13}C, ^{15}N]-labeled samples were grown in Silantes CN OD2 medium (www.silantes.com, distributed in US by Cambridge Isotope Laboratories, Inc.). All lanes were loaded with the equivalent to 10 µl of cell culture and run on 4–20% Tris-Glycine gels (Invitrogen). Lane M, Broad Range Protein Molecular Weight Markers (Cat.# V8491); lane 1, before induction; lanes 2 and 5, total protein; lanes 3 and 6, induced lysate supernatant; lane 7, flowthrough from HisLink™ Resin; and lanes 4 and 8, elution from HisLink™ Resin. NMR data displayed in Panels B and C were recorded at the National Magnetic Resonance Facility at Madison (NMRFAM) on a 600 MHz Varian INOVA spectrometer equipped with a cryogenic ^{1}H, ^{15}N, ^{13}C triple-resonance probe. Concentration was estimated using A_{280} on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). **Panel B.** ^{1}H-^{15}N HSQC spectrum of ~1 mM [*U*-^{13}C, ^{15}N]-labeled CRBPII in 10 mM phosphate buffer (pH 6.8) with 130 mM NaCl, 25 °C, with 745 × 192 complex data points for proton and nitrogen, respectively. Four scans were accumulated for each increment. Total data collection time was 30 minutes. **Panel C.** ^{1}H-^{13}C HSQC spectrum of ~1 mM [*U*-^{13}C, ^{15}N]-labeled CRBPII in 10 mM phosphate buffer (pH 6.8) with 130 mM NaCl, 25 °C, with 745 × 256 complex data points for proton and carbon, respectively. Eight scans were accumulated for each increment. Total data collection time was 78 minutes.

[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.

[b] Patent Pending.

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