



## Selenomethionine Protein Labeling Using the *Escherichia coli* Strain KRX

**ABSTRACT** With Monster Green® fluorescent protein as a model, we can achieve an average of 90% or greater selenomethionine (Se-Met) incorporation when the GFP is expressed in the *E. coli* strain KRX. Therefore, it is feasible to produce Se-Met-labeled protein samples for structural biology applications, such as X-ray crystallography, using the Single Step (KRX) Competent Cells.

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**The KRX strain** was examined for its ability to incorporate Se-Met into a green fluorescent protein.

### INTRODUCTION

The highly competent Single Step (KRX) Competent Cells<sup>(a)</sup> can be used for high-efficiency cloning and tightly regulated recombinant protein production in *Escherichia coli* (1). The T7 RNA polymerase gene in the KRX strain is under the control of *rha*P<sub>BAD</sub> promoter, which is positively activated by rhamnose and catabolically repressed by glucose. Expression of target proteins under the T7 promoter can be achieved in KRX with very low basal level production as compared to DE3-based strains (1); thus, one might achieve high levels of specific incorporation of seleno-substituted amino acids [e.g., Se-Met and selenocysteine (Se-Cys)] and other stable isotopes (e.g., <sup>13</sup>C, <sup>15</sup>N) upon target protein induction.

Proteins overproduced in *E. coli* often are labeled

with Se-Met for structural analysis by X-ray crystallography. Se-Met incorporation is typically achieved in methionine auxotrophs but can also be successful for methionine autotrophs (2,3). We tested the applicability of KRX, a methionine autotroph, in producing Se-Met-labeled proteins by expressing Monster Green® fluorescent protein in the pFN6A (HQ) Flexi® Vector (Cat.# C8511). The target protein was purified using the HisLink™ Protein Purification Resin (Cat.# V8821) by virtue of its N-terminal HQ tag (4) and analyzed with MALDI-TOF mass spectrometry.

### SELENOMETHIONINE (SE-MET) LABELING

Se-Met incorporation into proteins can aid structure determination by X-ray crystallography using multi-wavelength anomalous diffraction (MAD).

**Table 1. Se-Met Labeling Protocol.**

<b>Day 1</b>
1. Inoculate 5ml of LB (supplemented with 0.4% glucose and 100mg/L ampicillin) with a single colony from freshly streaked plate.
2. Grow starter culture at 37°C for ~6–8 hours.
3. Inoculate 250ml of LB (supplemented with 0.8% glucose and 100mg/L ampicillin) using the 5ml starter culture.
4. Grow overnight at 37°C (<18 hours).
<b>Day 2</b>
5. Centrifuge the overnight culture at 3,000rpm (Beckman JA-14 rotor) for 10 minutes at 4°C to collect cells.
6. Resuspend cell pellet in 250ml (1:1 ratio to the overnight culture) of induction media [1X M9 media (10) with 1% (v/v) glycerol substituting for glucose, 1X metal mix (2) and 125mg/L Se-Met (Sigma)].
7. Incubate the cells at 25°C for 30 minutes to 1 hour.
8. Induce cells with 0.2% (w/v) rhamnose for 1–2 days at 25°C.
<b>Days 3–4</b>
9. Centrifuge cells at 5,000rpm (Beckman JA-14 rotor) for 20 minutes at 4°C.
10. Resuspend cell pellet in 50ml Falcon™ tubes using 25ml (1/10 volume of induction media) of 1X FastBreak™ Cell Lysis Reagent (Cat.# V8571) supplemented with 25µl of RQ1 RNase-Free DNase (Cat.# M6101; diluted 1:1,000) and protease inhibitor cocktail (Roche).
11. Incubate resuspended cells at room temperature for 20 minutes.
12. Centrifuge the lysed cells at 7,500rpm (Beckman JA-14 rotor) for 20 minutes at 4°C.
13. Transfer the supernatant onto a pre-equilibrated 1ml (1/250 volume of induction culture) HisLink™ Resin gravity column.
14. Wash column with 20ml (20X column volume) of binding/wash buffer (10mM HEPES [pH 7.5], 500mM NaCl, 10mM imidazole).
15. Elute protein with 10ml (10X column volume) of elution buffer (10mM HEPES [pH 7.5], 500mM NaCl, 500mM imidazole).

Hendrickson and colleagues first demonstrated the use of selenium as an anomalous scatterer (5), solved the structure of selenobiotyl streptavidin (6), then with selenomethionyl-substituted streptavidin (7). Thereafter, Se-Met incorporation into proteins has become a popular and powerful tool for X-ray crystallographers.

Before examining Se-Met labeling in KRX, we tested the expression of unmodified Monster Green® protein in Terrific broth, establishing the optimal post-induction harvest time as 1–2 days at 25°C when the cell culture turned bright green. However, Se-Met inhibits cell growth. To maximize protein yield, we developed a modified cell-condensation procedure (8,9) for Se-Met incorporation in minimal media with KRX. The protocol is summarized in Table 1.

The purified proteins were analyzed on 4–20% SDS-PAGE gels (Invitrogen). Quantification of protein yield was estimated by A<sub>280</sub> on a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). The molecular weight of purified proteins was determined by MALDI-TOF mass spectrometry (HT Laboratories, Inc.).

$$\% \text{ Labeling Efficiency} = \frac{100 \times (MW_{\text{labeled\_experimental}} - MW_{\text{unlabeled\_experimental}})}{(MW_{\text{labeled\_theoretical}} - MW_{\text{unlabeled\_theoretical}})}$$

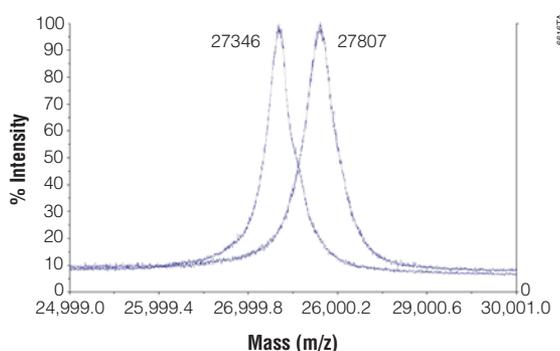
Labeling efficiency was calculated by the following formula, where MW is molecular weight in Daltons:

All samples gave efficient Se-Met labeling as summarized in Table 2. With three separate preparations of Se-Met-labeled Monster Green® protein, we saw an average Se-Met incorporation of ~93%, indicating that ~10.2 out of the possible 11 methionines (including the first methionine residue) were substituted by Se-Met. We had uniform labeling as shown by the overlay of MALDI-TOF traces in Figure 1, with no peaks correlating to the unlabeled protein in the Se-Met-substituted sample. This further validates the tight control of basal-level protein production in KRX.

**Table 2. Se-Met Incorporation Efficiency with 125mg/L of Se-Met in the Induction Media.**

Sample	Unlabeled (Da)	Se-Met (Da)	% Labeling	#Se Incorporated
1	27346	27807	89.3	9.8
2	27322	27865	100	11
3	27354	27820	90.3	9.9
Average	27341	27831	93.2	10.2

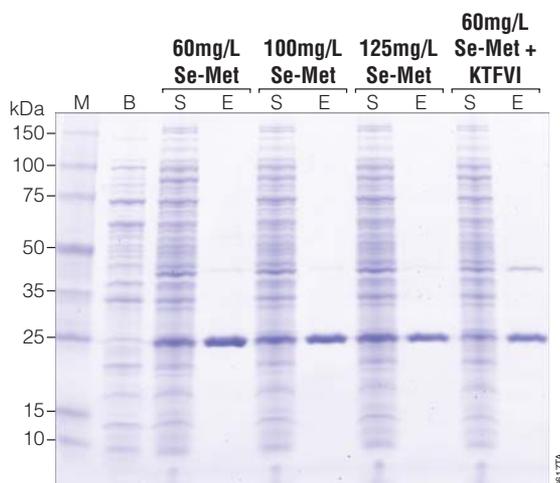
The molecular weight of purified proteins was determined by MALDI-TOF mass spectrometry (MW<sub>experimental</sub>). Theoretical value of unlabeled protein is 27,349Da, and of Se-Met fully substituted protein is 27,865Da as calculated by Protein Calculators v3.3 ([www.scripps.edu/~cdputnam/protcalc.html](http://www.scripps.edu/~cdputnam/protcalc.html)). There are a total of 11 methionines (including the N-terminal) in the protein. Se-Met was from Sigma. See Table 1 for growth, induction and purification protocol.



**Figure 1. Overlay of MALDI-TOF mass spectrometry traces of the unlabeled and Se-Met-labeled protein.** The protein samples were analyzed by HT laboratories, Inc. (San Diego, CA). The protein concentrations used for this analysis were approximately 0.3mg/ml.

**SELENOMETHIONINE TITRATION**

We performed a titration using 60, 100 and 125mg/L of Se-Met to determine the minimum requirement of Se-Met for labeling. Since methionine synthesis pathway inhibition is often used for Se-Met incorporation with methionine autotrophs (3), we also included an induction culture of 60mg/L Se-Met with 100mg/L each of lysine, threonine and phenylalanine, and 50mg/L each of valine and isoleucine (KTFVI) inhibit endogenous methionine biosynthesis. The protein was expressed and purified as described in Table 1 and analyzed by SDS-PAGE (Figure 2).



**Figure 2. Se-Met titrations of Monster Green® protein expression.** Fifteen microliters of cell culture for each Se-Met titration value (60–125mg/L) was analyzed by 4–20% Tris-glycine SDS-PAGE (Invitrogen) stained with Coomassie® Blue. Titration was performed as described in Table 1. Lane B, before induction; Lane S, soluble lysate; lane E, elution from HisLink™ Protein Purification Resin; lane M, Broad Range Protein Molecular Weight Markers (Cat.# V8491).

**An average ~93% Se-Met incorporation translates to ~10.2 out of the possible 11 methionines in Monster Green® protein substituted with Se-Met.**

**Table 3. Se-Met Incorporation Efficiency After Se-Met Titration in the Induction Media.**

Sample	Se-Met (mg/L)	Se-Met (Da)	% Labeling	#Se Incorporated
1	60	27838	100	11
2	100	27849	100	11
3	125	27801	95.5	10.5
4	60+KTFVI	27783	90.1	9.9
5	unlabeled	27318	–	–

The molecular weight of purified proteins was determined by MALDI-TOF mass spectrometry ( $MW_{\text{experimental}}$ ). Theoretical value of unlabeled protein is 27,349Da, and of Se-Met fully substituted protein is 27,865Da as calculated by Protein Calculators v3.3 ([www.scripps.edu/~cdputnam/protcalc.html](http://www.scripps.edu/~cdputnam/protcalc.html)). There are a total of 11 methionines (including the N-terminal) in the protein. Se-Met was from Sigma. See Table 1 for growth, induction and purification protocol.

**The Single Step (KRX) Competent Cells** can be used to both clone and express a protein for efficient Se-Met incorporation.

We analyzed Se-Met incorporation further using MALDI-TOF mass spectrometry and summarized the results in Table 3. For this experiment, the incorporation of Se-Met into Monster Green<sup>®</sup> protein was efficient with 60mg/L of Se-Met in the induction media. Including the amino acid mix KTFVI did not increase Se-Met incorporation.

**CONCLUSIONS**

We have demonstrated that the Single Step (KRX) Competent Cells, with low basal level of protein production, is suitable for applications that require efficient Se-Met incorporation. Therefore, Single Step (KRX) Competent Cells can be used to clone, express and Se-Met-label proteins without the need to transfer the expression plasmid into another expression/labeling strain.

**PROTOCOL**

- *Single Step (KRX) Competent Cells Technical Bulletin #TB352*, Promega Corporation  
[www.promega.com/tbs/tb352/tb352.html](http://www.promega.com/tbs/tb352/tb352.html)

**REFERENCES**

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**ORDERING INFORMATION**

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

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