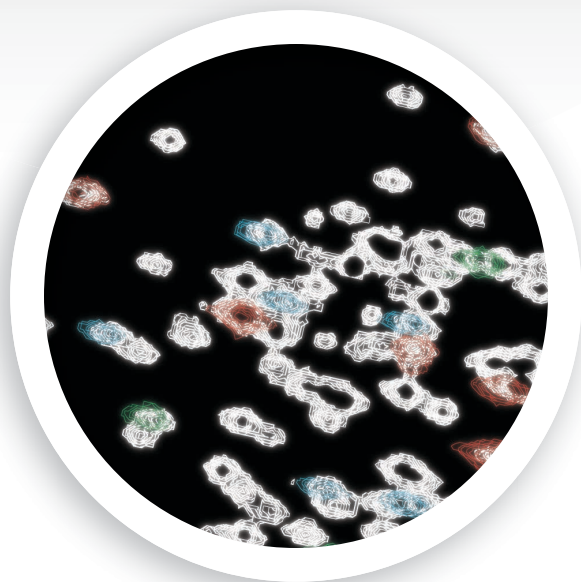


Efficient Uniform Labeling of Proteins Expressed in Baculovirus-Infected Insect Cells Using BioExpress® 2000 (Insect Cell) Medium

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Uniform isotope labeling is a key tool for NMR studies on recombinant proteins and their interaction with ligands of pharmaceutical interest. For this purpose, most recombinant proteins have been expressed in labeled form using *E. coli*. However, such expression is restricted to proteins of a non-complex nature. As expression of more complex proteins is routinely done in cell cultures of higher eukaryotes, using mammalian or insect cells, the need for isotope-labeling methods for these expression systems is evident. The recent availability of a suitable labeling medium, BioExpress® 2000 (insect cells) from Cambridge Isotope Laboratories, Inc., (CIL) makes it possible to efficiently isotope label more complex proteins, such as kinases expressed in baculovirus-infected insect cells (Strauss, et al., 2005). This labeling medium is commercially available from CIL in three different forms: "BioExpress® 2000 (insect cell) (unlabeled)" (CIL #CGM-2000-U), called here BioExpress® 2000-U; "BioExpress® 2000 (insect cell) (¹⁵N-labeled)" (CIL #CGM-2000-N), called here BioExpress® 2000-N; "BioExpress® 2000 (insect cell) (¹³C/¹⁵N-labeled)" (CIL #CGM-2000-CN), called here BioExpress® 2000-CN.

The catalytic domain of Abl kinase (Abl) was used as model protein in this study because Abl is a typical tyrosine kinase, is well expressed in baculovirus-infected insect cells, information on the crystal structure and on amino acid-specific isotope labeling is known (Strauss, et al., 2003) and furthermore it is a therapeutically relevant target.

Successful uniform isotope labeling of proteins with baculovirus-infected insect cells is dependent on:

- good expression of soluble and correctly folded protein in the labeling medium;**
- a suitable labeling medium giving sufficiently high label incorporation;**
- a suitable culture method compatible with labeling.**

To be useful for most important NMR studies, a labeled protein has to be expressed with baculovirus-infected insect cells in the labeling medium in sufficient quality and quantity for isolation of at least 10 mg correctly folded protein. This can be easily achieved for Abl kinase with half a liter of labeling medium. Upon re-suspension of

(continued)

cells and expression in BioExpress® 2000, Abl is the most abundant protein under these culture conditions as shown by SDS-PAGE analysis (Figure 1A). Expression levels were as high as 115 mg/L total Abl protein (Table 1) and 50-80 mg/L of isolated protein (Table 2). Similarly high expression under these conditions was also observed in BioExpress® 2000 for another kinase (KDR) and two other proteins expressed in the baculovirus system (Figure 1A), as well as several other proteins tested for expression (data not shown). For all these proteins, expression levels and cellular yield in expression cultures of Sf9 cells re-suspended in BioExpress® 2000 were similar to those achieved using “standard” expression media

such as SF900 II (Invitrogen) or EX-CELL 420 (JRH) (Figure 1A, Table 1). For obtaining these and the following results on expression of Abl, 16 μ M STI571, a specific Abl kinase inhibitor, was added to the culture at infection in order to reduce phosphorylation and stabilize the protein (Strauss, et al., 2005).

Apart from allowing good expression of a recombinant protein, an isotope-labeling medium for BV-infected insect cells suitable for advanced NMR studies has to contain all 20 amino acids isotopically labeled at a high percentage to ensure high label incorporation into the recombinant protein. This is the case for both, the ^{15}N -labeling

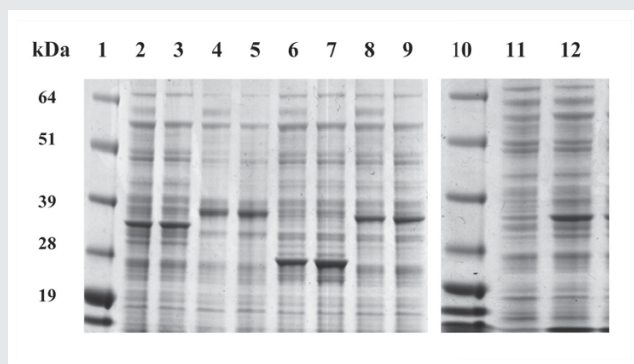


Figure 1. SDS-PAGE analysis by Coomassie blue staining of total lysates from BV-infected Sf9 cells expressing recombinant proteins, cultured in 50 mL shake flask cultures. Lanes 1 and 10 show molecular weight marker proteins (Invitrogen, SeeBlue Plus2).

- A: Expression of Abl kinase (6His-TEV-GAMPD-Abl/S²²⁹-S⁵⁰⁰) in presence of 16 μ M STI571 (lanes 2 and 3), KDR kinase (M⁸⁰⁶-K⁹⁴¹/D50/P⁹⁹²-D¹¹⁷¹-ThrcS-6His) in presence of 10 μ M PTK⁷⁸⁷ (lanes 4 and 5), a nuclear receptor protein (lanes 6 and 7) and another kinase (lanes 8 and 9) in Sf9 cultures after growth in SF900 II medium and medium change to labeling medium BioExpress® 2000-U (lanes 2, 4, 6 and 8) or to expression medium SF900 II (lanes 3, 5, 7 and 9).
- B: Expression of Abl kinase in presence of 16 μ M STI571 after growth of Sf9 cells and expression in BioExpress® 2000-U without medium change (lane 11) or growth in SF900 II and medium change to BioExpress® 2000-U (lane 12).

Culture Medium	Cell density (x10 ⁶ /mL)	Viability (%)	Fresh weight (g/30mL)	Cell diameter (mm)	Abl expression (mg/L)
BioExpress® 2000-U	1.60±0.09	93.3±1.7	0.50±0.01	24.0±0.8	115±5
SF900 II	1.74±0.22	90.3±1.4	0.51±0.03	24.3±1.1	136±26
EX-CELL 420	1.60±0.06	89.9±1.4	0.56±0.03	24.3±1.6	135±24

Table 1. Cell density, viability, yield, diameter and Abl expression levels of Sf9 cells cultured in 50 mL shake flasks, three days post-infection in labeling medium BE2000-U compared to standard expression media after growth in SF900 II and medium change prior to BV infection. Cell density, viability and diameter are determined by a Vi-CELL XR (Beckman Coulter) cell counter. Abl expression is quantified by gel-densitometric determination of the fraction of Abl in relation to the total protein content (determined by the Bradford method). Mean values from three parallel cultures and the resulting standard deviations are given.

Labeling type of Abl (Medium)	Isolated 6His-Abl protein ¹⁾	Label incorporation rate ²⁾	Performed NMR analysis
Unlabeled (BioExpress® 2000-U)	26mg/0.44L	-	-
Uniform ^{15}N -labeling (BioExpress® 2000-N)	16mg/0.24L	91.4 %	^1H - ^{15}N -HSQC spectrum
Uniform $^{13}\text{C}/^{15}\text{N}$ -labeling (BioExpress® 2000-CN)	40mg/0.47L	90.5 %	^1H - ^{15}N -HSQC spectrum

Table 2. Yield of isolated 6His-Abl protein, label incorporation rates and performed NMR studies from liter-scale productions of isotope-labeled Abl kinase expressed in rec. BV-infected Sf9 cells.

- 1) 6His-Abl eluted from Ni-NTA column measured by HPLC.
2) Label incorporation rates are calculated as percentage of the observed to the theoretical mass increase in MS spectra of purified unphosphorylated Abl samples.

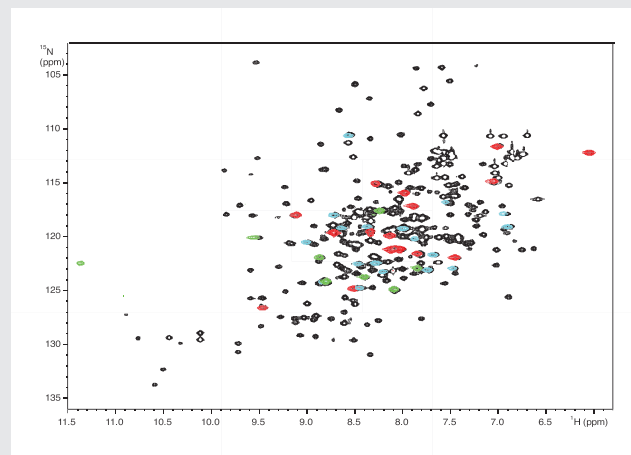


Figure 2. [^{15}N ; ^1H]-HSQC spectrum for uniformly ^{15}N -labeled Abl kinase (in black) expressed in BV-infected Sf9 cells, superimposed with the resonances from Abl kinase labeled with ^{15}N -Phe (in green), ^{15}N -Tyr (in red), ^{15}N -Val (in blue) (from Strauss, et al., 2003). One resonance at 133.4 ppm (^{15}N) / 12.6 ppm (^1H) lies outside the boundaries of the figure.

medium (BioExpress® 2000-N) and the $^{13}\text{C}/^{15}\text{N}$ -labeling medium (BioExpress® 2000-CN), where high incorporation rates of 91.4% and 90.5%, respectively, have been found for Abl kinase expressed in BV-infected Sf9 cells (Table 2; Strauss, et al., 2005).

For $^{13}\text{C}/^{15}\text{N}$ -labeling of Abl in BioExpress® 2000-CN, it was shown (Strauss, et al., 2005) that the carbohydrates have to be present in ^{13}C -labeled form to avoid label dilution. With ^{15}N -labeled Abl and $^{13}\text{C}/^{15}\text{N}$ -labeled Abl, high quality ^1H - ^{15}N -HSQC spectra with identical resonance patterns were obtained upon NMR analysis. The majority of the 277 amino acids of GAMDP-Abl (229-500) show up as clear and defined resonances as shown at the example of the ^1H - ^{15}N -HSQC spectrum of ^{15}N -labeled Abl (Figure 2). The same is true for the ^1H - ^{15}N -HSQC spectrum of $^{13}\text{C}/^{15}\text{N}$ -labeled Abl (Strauss, et al., 2005).

The most suitable culture protocol for uniform isotope labeling of a recombinant protein involves an initial growth phase for the insect cells in an unlabeled growth/expression medium and subsequently an expression phase where cells have been transferred by centrifugation to the labeling medium, prior to infection with the recombinant BV. This protocol allows much higher expression levels of the labeled protein (as shown in Figure 1B) compared to one with growth and expression in labeling medium without medium change.

A summarized version of the successful protocol for uniform isotope labeling of Abl described above is given in Figure 3. General methods for working with insect cells and baculovirus are given in several textbooks (e.g. O'Reilly, et al., 1994). A more detailed description of the experimental protocol on uniform isotope labeling of Abl in BV-infected insect cells is given elsewhere (Strauss, et al., 2005).

This uniform isotope labeling should also be applicable to other insect cell lines, as high expression of Abl in BioExpress® 2000-U with Sf21 or H5 cells was also achieved.

In conclusion, the method described opens up the possibility of efficient uniform isotope labeling of more complex recombinant proteins expressed in baculovirus-infected insect cells.

BioExpress® is a registered trademark of Cambridge Isotope Laboratories, Inc.

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Protocol for uniform isotope labeling of proteins with BV-infected Sf9 cells

1. Prior to performing isotope labeling of a protein, optimize culture and BV-infection conditions in unlabeled labeling medium (e.g. BioExpress® 2000-U) for expression of the protein.
2. Several 100 mL cultures of Sf9 cells adapted to growth in serum-free medium SF900 II in 500 mL Erlenmeyer flasks are cultivated for 3 days at 27°C, shaken at 90 rpm.
3. Prepare the uniform isotope-labeling medium (e.g. BioExpress® 2000-CN) according to CIL's instructions. It can be stored, filter-sterilized, for several months at 4°C in the dark without loss of capacity for protein expression. Requires warming up to 28°C before use.
4. When the final cell density of the culture has reached $\sim 1.5 \times 10^6$ c/mL (~ 3 days), sterile centrifuge the cells at 400G for 20 minutes at 20°C.
5. Re-suspend the pelleted cells in 100 mL portions in labeled medium (e.g. BioExpress® 2000-CN) and transfer to fresh 500 mL Erlenmeyer flasks.
6. Add the recombinant baculovirus of a titer of $0.5 - 2 \times 10^8$ pfu/mL to a MOI=1-2, according to optimized conditions.
7. The 100 mL cultures of BV-infected Sf9 cells are grown for three days in labeled medium post infection at 27°C, shaken at 90 rpm.
8. Harvest the cells expressing the labeled recombinant protein by centrifugation (400G, 20 minutes, at 20°C); re-suspend the pelleted cells in PBS, pH 6.2 with protease inhibitor mix (Complete™, Roche) followed by a second centrifugation in 50 mL plastic tubes under conditions as above. Store the pelleted cells at -80°C.
9. Isolate and purify the recombinant protein according to protocols generated for the unlabeled protein. MS and NMR analysis are carried out for proteins labeled in *E. coli*.

Figure 3. Recommended protocol for successful uniform isotope labeling of recombinant proteins with BV-infected Sf9 cells. This protocol was in general followed for obtaining the herein presented data. For a successful uniform isotope labeling of a protein in BioExpress®-2000 with BV-infected insect cells as shown above for Abl kinase, the prerequisites listed in Figure 4 must be fulfilled.

Prerequisites for uniform isotope labeling of a protein with BV-infected insect cells in labeling media of the BioExpress® 2000 series

- The protein to be labeled must be <35 kDa in size and expressed in insect cells in BioExpress® 2000-U as mainly soluble, correctly folded, monomeric and sufficiently stable protein in amounts >10 mg/L.
- A culture volume with BioExpress® 2000-N or BioExpress® 2000-CN for uniform isotope labeling of a protein sufficient for the isolation of >10 mg of purified, correctly folded labeled protein should be chosen.
- Use a successful labeling protocol as given above in Figure 3.

Figure 4. Prerequisites for successful uniform isotope labeling of a protein with BV-infected insect cells.

