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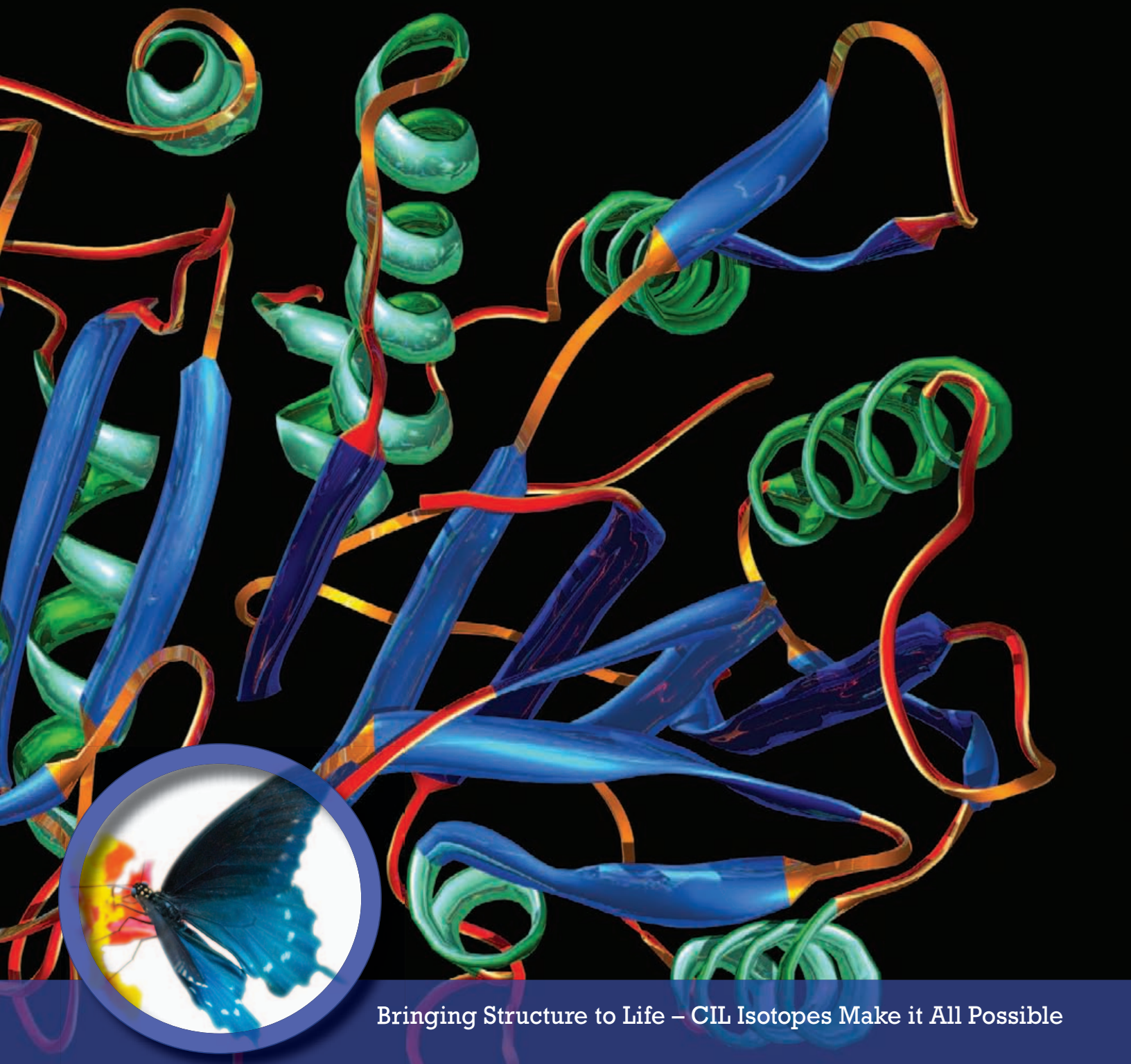
Cambridge Isotope Laboratories, Inc.

Stable Isotopes for Structural Biomolecular NMR



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Welcome

Kevin Millis, Ph.D.
Cambridge Isotope Laboratories, Inc.

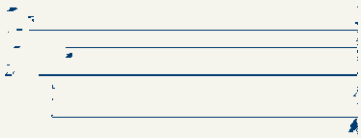
It is with great pride that we present CIL's new Biomolecular NMR Product Catalog. In this catalog you will find a comprehensive listing of our isotope-enriched products used to study the structure and dynamics of protein and nucleic acids using nuclear magnetic resonance.

We are pleased to present contributions written from some of the world's most preeminent NMR spectroscopists. We are particularly pleased to present pieces written by Gerhard Wagner, Stanley Opella, Lewis Kay, Masatsune Kainosho, Leonid Brown & Vladimir Ladizhansky, Hanudatta Atreya, and Linda Columbus & Daniel Fox.

Included in this catalog are a collection of CIL application notes relevant to magnetic resonance studies of biological molecules and systems. These application notes help illustrate the usefulness of our products for both solution and solid-state NMR applications. We would like to thank the many researchers who have written application notes that are included in this catalog. Also included in this catalog is our most recent application note containing previously unpublished data that was written by Drs. Leonid S. Brown and Vladimir Ladizhansky (Application Note 26). These methods provide additional tools to study the structure and behavior of membrane proteins.

As new technologies and applications to study biopolymers using NMR advance, CIL continues to maintain a leadership role in developing new products for the benefit of the entire biomolecular NMR community. CIL welcomes suggestions of our customers on additional products which would be beneficial to their research. It has been through our partnerships and close relationships with our customers over the past 30 years that we have been able to significantly expand our product offering in order to assist the biomolecular NMR community in the advancement of their studies utilizing stable isotope-labeled compounds as a tool to structural NMR discovery.

All of us as CIL appreciate the collaborative relationships that we have with our customers. As the CIL Biomolecular NMR Product Manager, I look forward to continuing to work with you to expand our product offerings in ways that will best fulfill your needs.



Kevin Millis, Ph.D.
CIL Biomolecular NMR Product Manager

Cambridge Isotope Laboratories (CIL) is the world leader in the separation and manufacture of stable isotopes and stable isotope-labeled compounds. For over 30 years CIL has remained the premier supplier of stable isotopes for NMR and MRS/MRI research applications. These products include a diverse line of RNA/DNA products, minimal media reagents (carbohydrates, ammonium salts), cell-free expression reagents and kits, free and protected amino acids, as well as cell-growth media for eukaryotic and prokaryotic cell lines. Additionally, CIL offers a comprehensive line of deuterated solvents, detergents and buffers. Our products have been specifically designed and tested with the most discerning NMR spectroscopist and structural biologist in mind. CIL actively supports the world's NMR community through meeting sponsorships and customer collaborations.

Cambridge Isotope Laboratories' area of expertise is the labeling of biochemical and organic compounds with highly enriched isotopes of carbon, hydrogen, nitrogen and oxygen. CIL is committed to providing the highest quality products coupled with unsurpassed service to researchers worldwide. With more than 10,000 products in our combined Research Products and Environmental Contaminant Standards inventory, we proudly offer the world's largest range of stable isotope-labeled compounds. For more than 30 years, CIL products have contributed to advancements in drug discovery, environmental analysis, genomics and proteomics, and medical diagnostic research. We are proud of our history and our past success, which have been made possible by the talent and commitment of our employees. We look forward to continuing to service the scientific research community as we develop new stable isotope-labeled tools to aid researchers with new discoveries.

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Utility of Stable Isotopes in Structural Biology

Gerhard Wagner, Ph.D.
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts

The use of stable isotopes has empowered NMR to become a major player in mechanistic structural biology and drug discovery. Over the past quarter century introduction of new isotope-labeling procedures has led to several quantum leaps in the advancement of NMR spectroscopy. By introducing new NMR active nuclei, or replacing protons with deuterons, isotope labeling is used to obtain new correlations and to reduce complexity of spectra. Thus, it has tremendously facilitated obtaining specific structural and dynamic information. New isotope-labeling procedures have stimulated the development of a plethora of new NMR experiments. Today, labeling is an integral element of current state-of-the-art NMR technology and can be expected to play a major role in future developments. While new labeling methods typically originate from academic laboratories, it is usually not economical to cultivate procedures for every isotope-producing procedure in all interested laboratories with student and post-doc members. This is best pursued by companies, such as CIL, that can provide sustainable long-term isotope commodities to the scientific community and are an invaluable service for the NMR community.

Until the early 1970's NMR spectroscopy was a method primarily used by chemists for characterizing small molecules, checking chemical structures and purity of reaction products. Studies of proteins and nucleic acids became feasible with the arrival of high-resolution superconducting magnets at higher field and new consoles for pulsed NMR, development of double-resonance experiments, intelligent use of the nuclear Overhauser effect, and eventually the introduction of 2D NMR. This allowed collection of numerous structural parameters, and with the development of new computer programs, the first solution structures were determined with NMR in the early to mid 1980's. However, the method was limited to proteins up to 10 kDa or 15 kDa, and the process was very time consuming.

Fortunately, techniques were developed for producing isotope-labeled proteins in bacterial expression systems.[1,2] The availability of ^{15}N labeled proteins enabled a first quantum leap. Three-dimensional ^{15}N-dispersed NOESY experiments were developed leading to a tremendous gain in signal dispersion and spectral simplification. Larger structures could be obtained in shorter time and with higher precision. Access to routine production of ^{15}N and ^{13}C-labeled proteins has also opened an entirely new field of protein dynamics, which is thriving ever since. It has provided and is yielding new insights into how proteins fluctuate and perform functions, such as in enzymes.

The possibility to produce $^{15}N/^{13}C$ labeled proteins has made possible the development of triple-resonance experiments for conformation-independent sequential assignments. These experiments caused a second quantum leap in protein NMR and made assignments more reliable and very efficient. Triple resonance experiments form today the basis of all assignments of proteins.

Another quantum leap was the introduction of deuteration by over-expressing proteins in $^{2}H_2O$, which reduces dipolar broadening of resonances by proton dilution.[3] Spectroscopy is essentially done with the amide protons that can be reintroduced by dissolving deuterated proteins in H_2O for measurements. The reduction in line-widths of amide proteins was dramatic and excited everyone in the field when this was introduced. Deuteration is a key requirement for the performance of TROSY (Transverse Relaxation Optimized Spectroscopy) experiments, which brought another performance leap and is today used for nearly all NMR studies of larger proteins.

With deuterated proteins, backbone assignments are rather routine even for rather large proteins. The challenge is to have a sufficiently large number of probes for measuring distance constraints. Thus, introduction of selectively protonated groups into deuterated proteins has become very important. Most prominent is the introduction of protonated ILV methyls, which uses ^{13}C α-ketoisovalerate and α-ketobutyrate as precursors for labeling. There is a wide range of applications to introduce protonated groups into deuterated proteins beyond ILV labeling. Most sophisticated is the SAIL (Stereo-Arrayed Isotope Labeling) approach, which minimizes dipolar broadening while maintaining a large number of protons for distance measurements.

In our laboratory, the use of ILV labeling in a deuterated background, spiked with few protonated residues has allowed solving several large structures, where the prime source of distance constraints came from methyl-methyl and methyl-aromatic contacts. The clear separation of the methyl signals from the amides as the only protons made possible recording multiple high-resolution 4D NOESYs in a time-shared manner within a reasonable time.[4] Figure 1 shows the structure of the enterobactin synthase 37 kDa EntF T-TE di-domain where most distance restraints were obtained from such isotope-labeled samples.[5]

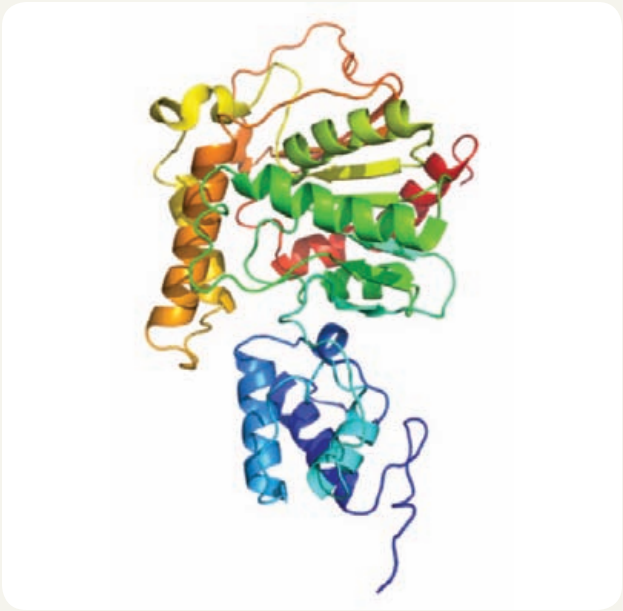

Figure 1. Structure of the 37 kDa EntF T-TE di-domain.[5]

Recent developments have explored additional possibilities for the use of isotopes to enhance NMR spectroscopy. This includes the advent of new cryogenic probes that are optimized for ^{13}C and ^{15}N direct detection. First used for direct detection of carbonyl carbons, direct detection seemed primarily suited for paramagnetic proteins where carbons could be observed closer to the paramagnetic center than protons. However, it became obvious that direct ^{13}C detection can be quite powerful for any protein when using alternate ^{13}C-^{12}C labeling, which can be obtained when using [2-^{13}C] or [1,3-$^{13}C_2$] glycerol as a carbon source as originally described by LeMaster.[6] This eliminates most one-bond carbon-carbon couplings. When the protein is deuterated in addition, carbon resonances become extremely sharp and new long-range correlations can be observed through backbone and side chain nuclei.[7,8] This is particularly interesting for assigning prolines, which are often found adjacent to phosphorylation sites in regulatory domains of proteins and are difficult to characterize with traditional NMR methods. It is likely that future developments of isotope labeling will enhance this approach.

Currently, the biggest hurdle in isotope-labeling proteins for NMR studies is the lack of efficient methods for producing isotope labeled proteins in eukaryotic or mammalian expression systems. Many mammalian proteins of interest are misfolded when expressed in bacterial systems due to the lack of folding machinery that is present in eukaryotic cells, such as those for forming disulfides. Production in insect cells, yeast or CHO (Chinese Hampster Ovary) cells is possible using special growth media, but the process is still far from being efficient for wide use.

Compared to proteins, the use of isotopes has been slower in NMR of nucleic acids. Due to a lower proton density, dipolar broadening is less of a problem in nucleic acids. Also connectivity through phosphorus is inefficient because of the large chemical shift anisotropy. Thus, assignments are mainly made with NOE correlations. However, novel labeling methods[9] and intelligent use of inserting deuterated nucleotides has made tremendous impact for structural studies of large nucleic acids.[10]

Considering the great impact isotope labeling had in the development of NMR in the past, it can be anticipated that it will play an equal or even greater role in the future.

Acknowledgement

Large amounts of stable isotopes were purchased with support from the NIH grant 047467.

References

1. LeMaster, D.M.; Richards, F.M. **1985**. Proton-nitrogen-15 heteronuclear NMR studies of *Escherichia coli* thioredoxin in samples isotopically labeled by residue type. *Biochemistry, 24*, 7263-7268.
2. McIntosh, L.P.; Dahlquist, F.W.; Redfield, A.G. **1987**. Proton NMR and NOE structural and dynamic studies of larger proteins and nucleic acids aided by isotope labels: T4 lysozyme. *J Biomol Struct Dyn, 5*, 21-34.
3. LeMaster, D.M. **1989**. Improved large scale culture of Methylophilus methylotrophus for ^{13}C/^{15}N labeling and random fractional deuteration of ribonucleotides. *Methods Enzymol, 177*, 23-43.
4. Frueh, D.P.; Vosburg, D.A.; Walsh, C.T.; Wagner, G. **2006**. Non-uniformly sampled double-TROSY hNcaNH experiments for NMR sequential assignments of large proteins. *J Biomol NMR, 34*, 31-40.
5. Frueh, D.P.; Arthanari, H.; Koglin, A.; Vosburg, D.A.; Bennett, A.E.; Walsh, C.T.; Wagner, G. **2008**. Dynamic thiolation-thioesterase structure of a non-ribosomal peptide synthetase. *Nature, 454*, 903-906.
6. LeMaster, D.M. **1996**. Protein Structure Refinement Using $^{13}C\alpha$ Chemical Shift Tensors. *J Am Chem Soc, 118*, 9255-9264.
7. Takeuchi, K.; Ng, E.; Malia, T.J.; Wagner, G. **2007**. 1-^{13}C amino acid selective labeling in a $^{2}H^{15}N$ background for NMR studies of large proteins. *J Biomol NMR, 38*(1), 89-98.
8. Takeuchi, K.; Frueh, D.P.; Sun, Z.Y.; Hiller, S.; Wagner, G. **2010**. HNCA-TOCSY-CANH experiments with alternate ^{13}C-^{12}C labeling: a set of 3D experiment with unique supra-sequential information for mainchain resonance assignment. *J Biomol NMR, 47*, 55-63.
9. Thakur, C.; Sama, J.; Jackson, M.; Chen, B.; Dayie, T. **2010**. Selective ^{13}C labeling of nucleotides for large RNA NMR spectroscopy using an *E. coli* strain disabled in the TCA cycle. *J Biomol NMR, 48*, 179-192.
10. Lu, K.; Miyazaki, Y.; Summers M.F. **2010**. A topical issue: production and labeling of biological macromolecules for NMR investigations *J Biomol NMR, 46*, 113-125.

Minimal Media Reagents

The *E. coli* expression system using minimal media is the most popular means to over-express isotope-enriched, recombinant protein for NMR investigations. The primary reason for the popularity of the use of minimal media is that it remains to be the most economic media available to grow isotope-labeled bacteria. Minimal media is a mixture M9 salts, glucose (as the sole carbon source) and ammonium chloride or ammonium sulfate (as the sole nitrogen source) that is formulated in either H_2O or D_2O. Often, depending on the *E. coli* strain used, additional reagents, such as cofactors, are added to the media to enhance growth.

For proteins greater than ~25 kDa in size, deuteration is generally required in order to simplify spectra and reduce the deleterious effects of line-broadening associated with ^1H dipolar coupling. Therefore, minimal media used to express such proteins must be formulated using D_2O. For the investigations of large proteins or protein complexes, fully deuterated glucose as the carbon source is required. In particular, it is advantageous to use deuterated glucose in conjunction with the ILV selective methyl-labeling strategies that require a highly deuterated protein. The use of protonated U-$^{13}C_6$ glucose as the carbon source may still be acceptable in some situations because the level of incorporation of deuterium from the solvent into the expressed proteins is still significant (*e.g.*, ~70-80%). The use of U-$^{13}C_6$ glucose in minimal media formulated in D_2O may also be used to label all protein methyl groups,[1] although CHD_2-detected variants must be used to remove signals from the CH_3 and CH_2D isotopomers in ^1H-^{13}C correlation spectra.

To aid in handling multiple simultaneous growths, auto-induction methods have been developed which utilize minimal media containing glucose, glycerol and lactose. Auto-induction methods utilize the preference of *E. coli* containing *lac* operon derived expression systems (*e.g.*, pET, pGEM, and pQE) to selectively use different carbon sources during diauxic growth. Hence auto-induction methods will lead to growth on alternative carbon sources such as either glycerol or lactose after exhaustion of glucose in the growth medium. The basis for auto-induction and adaptation to ^{13}C,^{15}N was reported by Studier[2] and further optimized for high-throughput production by Tyler, *et al.*[3]

Although minimal media offers the most economic media used to label *E. coli*, the resulting growth rate and expression levels are sometimes not suitable enough to conduct the desired studies. An economic method to enhance protein yield and growth characteristics is to spike low levels of suitably labeled rich media into minimal media prior to induction.*

The table below shows which labeled reagents to use in minimal media to obtain the highest level of incorporation of the desired labeling pattern.

Minimal Media Reagents used to Express Single-, Double- and Triple-Labeled Protein

Desired Labeling Pattern	^{15}N NH_4Cl or $(^{15}NH_4)_2SO_4$	D-Glucose (U-$^{13}C_6$, 99%)	D-Glucose ($^{13}C_6$, 99%; D_7, 98%)	D-Glucose (U-D_7, 98%)	D_2O
^{15}N	+	-	-	-	-
^{13}C	-	+	-	-	-
D	-	-	-	+	+
^{13}C,^{15}N	+	+	-	-	-
^{15}N, D	+	-	-	+	+
^{13}C, D	-	-	+	-	+
^{13}C,^{15}N, D	+	-	+	-	+

Minimal Media Reagents

Catalog No.	Description
CLM-1396	D-Glucose (U-$^{13}C_6$, 99%)
DLM-2062	D-Glucose (1,2,3,4,5,6,6-D_7, 98%)
CDLM-3813	D-Glucose (U-$^{13}C_6$, 99%; 1,2,3,4,5,6,6-D_7, 97-98%)
CLM-1510	Glycerol ($^{13}C_3$, 99%)
DLM-558	Glycerol (D_8, 99%)
NLM-467	Ammonium chloride (^{15}N, 99%)
NLM-713	Ammonium sulfate ($^{15}N_2$, 99%)
DLM-4-99	Deuterium oxide (D, 99%)
DLM-4-99.8	Deuterium oxide (D, 99.8%)
DLM-4-1L	Deuterium oxide (D, 9 9%)

*See CIL Application Note 12 (page 45).

References

1. Otten, R.; Chu, B.; Krewulak, K.D.; Vogel, H.; Mulder, F. **2010**. Comprehensive and cost-effective NMR spectroscopy of methyl groups in large proteins. *J Am Chem Soc, 132*, 2952-2960.
2. Studier, F. W. **2005**. Protein production by auto-induction in high-density shaking cultures. *Protein Expr Purif,* 41(*1*), 207-34.
3. Tyler, R.C.; Sreenath, H.K.; Singh, S.; Aceti, D.J.; Bingman, C.A.; Markley, J.L.; Fox, B.G. **2005**. Auto-induction medium for the production of [U-^{15}N]- and [U-^{13}C, U-^{15}N]-labeled proteins for NMR screening and structure determination. *Protein Expr Purif, 40*, 268-278.

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α-Keto Acids and Methyl-Labeling Kits and Amino Acids

Protonated ^{13}C methyl groups in deuterated proteins are excellent probes for structure and dynamics, and are especially crucial to study high-molecular weight proteins and protein complexes. The popular ILV labeling strategy utilizes labeled bio-synthetic precursors to produce protein containing either uniformly or selectively labeled isoleucine, leucine and valine. α-Ketoisobutyrate is the precursor to Ile and may be used to label the δ1 methyl in Ile. α-ketoisovalerate is the precursor to Leu and Val and is used to label one or both methyl groups in these amino acids. Please see CIL Application Notes 16 (page 55) and 25 (page 67) for more information regarding this application.

CIL is proud to offer a large selection of α-ketobutyric and α-ketoisovaleric acids, along with 3-^{13}C pyruvate, for use in selective methyl and side-chain labeling. Uniform ^{13}C-labeled forms of the precursors are used in conjunction with $^{13}C_6$ glucose to produce uniform ^{13}C-labeled Ile and Leu.

CIL also offers kits of conveniently packaged reagents for labeling proteins with isoleucine, leucine, valine, and alanine (*i.e.*, "ILVA" labeling) or only alanine. These kits are for use with 1 L amounts of deuterated minimal media. Please see CIL Application Note 25 (page 67) for more details regarding ILVA and alanine labeling. CIL also is offering methionine (2,3,3,4,4-D_5, methyl-$^{13}CH_3$) for use with deuterated minimal media to provide a new methyl probe in addition to leu, Ile, Val, and Ala.

Alanine Probes of Supra-Molecular Structure and Dynamics

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The development of new protein labeling strategies, along with optimized experiments that exploit the label, have significantly impacted on the types of biochemical problems that can now be addressed by solution NMR spectroscopy. One popular strategy in studies of high molecular weight proteins involves the use of a pair of α-ketoacids, α-ketobutyrate and α-ketoisovalerate, which serve as the biosynthetic precursors for the production of Ile and Leu/Val, respectively.[1] Addition of these precursors to highly deuterated protein expression media produces U-^{2}H, Ile, Leu, Val- methyl-labeled proteins. These precursors are available with different methyl isotopomers ($^{13}CH_3$, $^{13}CH_2D$, $^{13}CHD_2$) so that a large variety of labeled proteins can be produced[2] and a correspondingly large number of experiments can be performed using ^{1}H, ^{13}C or ^{2}H nuclei.

Ile, Leu, Val methyl groups are powerful probes of side-chain structure and dynamics and their utility has been described in a significant number of papers.[2] Ala methyls, on the other hand, report on properties of the backbone and hence provide important complementary information. A number of recent publications outline approaches for the production of highly deuterated, Ala-[$^{13}CH_3$] labeled proteins.[3,4] Ala labeling is challenging since this residue is produced directly as a result of transamination of pyruvate, which is also a precursor in the production of the branched-chain amino acids. Transamination is reversible so even if free methyl-labeled Ala is provided to the media, scrambling will occur with label incorporated at a variety of potentially undesired locations. Recently Boisbouvier and coworkers have developed a procedure to generate methyl labeling at Ala side chains with minimal (<1%) scrambling.[3] This was achieved by adding 2-[^{2}H],3-[^{13}C]-Ala (800 mg/L) as well as precursors for other pathways in which the scrambled amino acids are produced.

The ability to produce highly deuterated, Ala-[$^{13}CH_3$] labeled proteins further increases the number of methyl probes available for studies of very high molecular weight systems.[5] A number of applications involving Ala methyl probes can be envisioned, including measurement of backbone dynamics through relaxation studies, probing structure via residual dipolar couplings, methyl-methyl NOEs (Nuclear Overhauser Effect) or PREs (Paramagnetic Relaxation Enhancement) and studies of molecular interactions.

References

1. Goto, N.K.; Gardner, K.H.; Mueller, G.A.; Willis, R.C.; Kay, L.E. **1999**. A robust and cost-effective method for the production of Val, Leu, Ile (δ1) methyl-protonated ^{15}N-, ^{13}C-, ^{2}H-labeled proteins. *J Biomol NMR, 13*, 369-374.
2. Tugarinov, V.; Kay, L.E. **2005**. Methyl groups as probes of structure and dynamics in NMR studies of high-molecular-weight proteins. *ChemBioChem, 6*, 1567-77.
3. Ayala, I.; Sounier, R.; Use, N.; Gans, P.; Boisbouvier, J. **2009**. An efficient protocol for the complete incorporation of methyl-protonated alanine in perdeuterated protein. *J Biomol NMR, 43*, 111-9.
4. Isaacson, R.L.; Simpson, P.J.; Liu, M.; Cota, E.; Zhang, X.; Freemont, P.; Matthews, S. **2007**. A new labeling method for methyl transverse relaxation-optimized spectroscopy NMR spectra of alanine residues. *J Am Chem Soc, 129*, 15428-9.
5. Amero, C.; Schanda, P.; Dura, M.A.; Ayala, I.; Marion, D.; Franzetti, B.; Brutscher, B.; Boisbouvier, J. **2009**. Fast two-dimensional NMR spectroscopy of high molecular weight protein assemblies. *J Am Chem Soc, 131*, 3448-9.

(continued)

α-Keto Acids and Methyl-Labeling Kits and Amino Acids

α-Keto Acids

Catalog No.	Description	Structures
CLM-6820	α-Ketobutyric acid, sodium salt (methyl-^{13}C, 99%)	$*CH_3CH_2COCOONa$
CLM-6164	α-Ketobutyric acid, sodium salt (U-$^{13}C_4$, 98%)	$*CH_3*CH_2*CO*COONa$
CDLM-4611	α-Ketobutyric acid, sodium salt ($^{13}C_4$, 98%; 3,3-D_2, 98%)	$*CH_3*CD_2*CO*COONa$
CDLM-7318	α-Ketobutyric acid, sodium salt (methyl-^{13}C, 99%; 3,3-D_2, 98%)	$*CH_3CD_2COCOONa$
CDLM-7353	α-Ketobutyric acid, sodium salt (4-^{13}C, 99%; 3,3,4,4-D_4, 98%)	$(*CHD_2)CD_2COCOONa$
CLM-6821	α-Ketoisovaleric acid, sodium salt (dimethyl-$^{13}C_2$, 99%)	$(*CH_3)_2CHCOCOONa$
CLM-4418	α-Ketoisovaleric acid, sodium salt (U-$^{13}C_5$, 98%)	$(*CH_3)_2*CH*CO*COONa$
CDLM-4418	α-Ketoisovaleric acid, sodium salt (U-$^{13}C_5$, 98%; 3-D_1, 98%)	$(*CH_3)_2*CD*CO*COONa$
CDLM-7317	α-Ketoisovaleric acid, sodium salt (3-methyl-^{13}C, 99%; 3,4,4,4-D_4, 98%)	$*CH_3CD(CD_3)COCOONa$
CDLM-7354	α-Ketoisovaleric acid, sodium salt (3-methyl-^{13}C, 99%; 3-methyl-D_2, 3,4,4,4-D_4, 98%)	$(*CHD_2)CD(CD_3)COCOONa$
CDLM-8100	α-Ketoisovaleric acid, sodium salt (1,2,3,4-$^{13}C_4$, 99%; 3,4',4',4'-D_4, 97-98%)	$*CH_3*CD(CD_3)*CO*COONa$
CDLM-8446	α-Ketoisovaleric acid, sodium salt (dimethyl-$^{13}C_2$, 98%; 3-methyl-D_2, 4,4-D_2, 98%)	$(*CHD_2)CH(*CHD_2)COCOONa$
CLM-2240	Sodium pyruvate ($^{13}C_3$, 99%)	$*CH_3*CO*COONa$

Methyl Labeling Amino Acids and Kits

Catalog No.	Description
CDLM-8649	L-Alanine (3-^{13}C, 99%; 2-D, 96%)*
New! CDLM-8885	L-Methionine (2,3,3,4,4-D_5, 98%; methyl-$^{13}CH_3$, 99%)
New! CDLM-8805-KIT	*In Vivo* Alanine Methyl Labeling Kit*
New! CDLM-8806-KIT	*In Vivo* ILVA Methyl Labeling Kit*

*See Application Note 25 (page 67)

New!

CDLM-8805-KIT – *In Vivo* Alanine Methyl Labeling Kit

This kit contains four separate vials of the following compounds in the amounts indicated.

Catalog No.	Compound Name	Amount in Vial
DLM-584	Succinic acid (D_4, 98%)	2.5 g
DLM-4646	α-Ketoisovaleric acid, sodium salt (U-D_7, 98%)	0.2 g
DLM-141	L-Isoleucine (U-D_{10}, 98%)	0.06 g
CDLM-8649	L-Alanine (3-^{13}C, 99%; 2-D, 96%)	0.8 g

The materials in this kit are to be used in conjunction with 1 L of deuterated minimal media.[1,2] 1 L of minimal media that is suitable for use with this product typically contains 2 g of D-glucose-D_7, 1 g of ammonium salt, and 11.3 g of M9 salts in D_2O.

New!

CDLM-8806-KIT – *In Vivo* ILVA Methyl Labeling Kit

This kit contains four separate vials of the following compounds in the amounts indicated.

Catalog No.	Compound Name	Amount in Vial
DLM-584	Succinic acid (D_4, 98%)	2.5 g
CDLM-7317	α-Ketoisovaleric acid, sodium salt (methyl-^{13}C, 99%; 3,4,4,4-D_4, 98%)	0.12 g
CDLM-7318	α-Ketobutyric acid, sodium salt (methyl-^{13}C, 99%: 3,3-D_2, 98%)	0.06 g
CDLM-8649	L-Alanine (3-^{13}C, 99%; 2-D, 96%)	0.8 g

The materials in this kit are to be used in conjunction with 1 L of deuterated minimal media. 1 L of minimal media that is suitable for use with this product typically contains 2 g of D-glucose-D_7, 1 g of ammonium salt, and 11.3 g of M9 salts in D_2O.

References

1. Ayala, I.; Sounier, R.; Use, N.; Gans, P.; Boisbouvier, J. **2009**. *J Biomol NMR*, *43*, 111-9.
2. Isaacson, R.L.; Simpson, P.J.; Liu, M.; Cota, E.; Zhange, X.; Freemont, P.; Matthews, S. **2007**. *J Am Chem Soc, 129*, 15428-9.

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Rich *E. coli* Media

Although growth in minimal (M9) media is economical, there is no substitute for the enhanced growth rates and increased levels of protein expression that may be gained by the use of a rich medium. Rich bacterial media are complex formulations that are usually derived from algal hydroylsates and contain all the necessary nutrients to promote excellent growth. CIL offers a number of rich media used in labeled protein expression using bacterial systems.

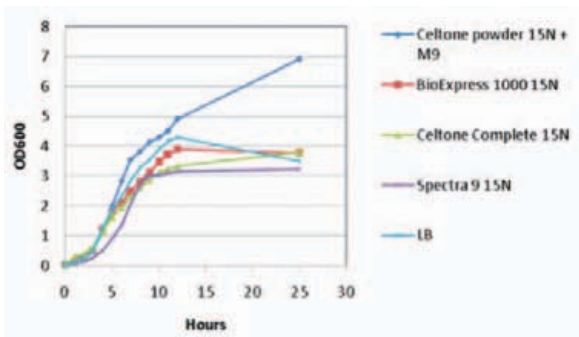
For pointers on how to maximize protein yield using CIL's BioExpress® 1000 media, please see CIL Application Note 15 (page 51). Please see CIL Application Note 12 (page 45) to learn how spiking BioExpress® 1000 media into minimal media provides a low-cost means to enhance the performance of minimal media.

BioExpress® 1000

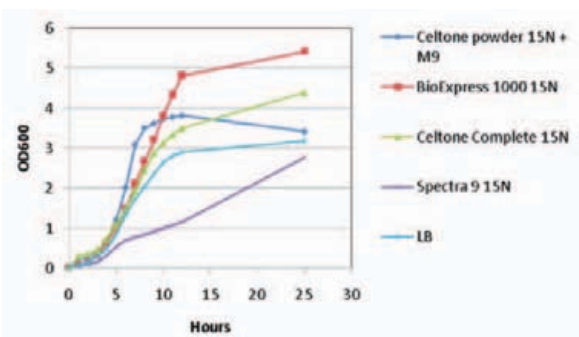
BioExpress® 1000 is CIL's all-time classic rich bacterial cell growth medium. BioExpress® 1000 provides excellent growth and expression characteristics for a number of different bacterial systems. BioExpress® 1000 contains nearly the same level of amino acids as LB medium. Glucose levels range from 0.1-0.5 g/L, depending on the batch. BioExpress® 1000 media is prepared by adding sterile cell-culture grade water and mixing. Please note that D_2O is required for reconstitution for products containing deuterium. BioExpress® 1000 is supplied as a 100 mL sterile liquid 10x concentrate, and reconstitutes to 1 L with no final pH adjustment required; 10 mL sample sizes are also available. The 10 mL sample size reconstitutes to make 100 mL of media with no final pH adjustment required.

Catalog No.	Description
CGM-1000-C	BioExpress® 1000 (U-^{13}C, 98%) (10x Concentrate)
CGM-1000-D	BioExpress® 1000 (U-D, 98%) (10x Concentrate)
CGM-1000-N	BioExpress® 1000 (U-^{15}N, 98%) (10x Concentrate)
CGM-1000-CD	BioExpress® 1000 (U-^{13}C, 98%; U-D, 98%) (10x Concentrate)
CGM-1000-CN	BioExpress® 1000 (U-^{13}C, 98%; U-^{15}N, 98%) (10x Concentrate)
CGM-1000-DN	BioExpress® 1000 (U-D, 98%; U-^{15}N, 98%) (10x Concentrate)
CGM-1000-CDN	BioExpress® 1000 (U-^{13}C, 98%; U-^{15}N, 98%; U-D, 98%) (10x Concentrate)
CGM-1000-U	BioExpress® 1000 Unlabeled (10x Concentrate)

Growth Curves *E. coli* (BL21, pGFP)



Growth Curves *E. coli* (M15, pTMK)



"In our hands, CIL's BioExpress® 1000 worked like a charm. The cell growth rate and protein expression level essentially matched the results obtained with Luria broth, and the ^{15}N labeling efficiency was excellent."

Dr. Tero Pihlajamaa
Institute of Biotechnology
University of Helsinki, Finland

(continued)

Rich *E. coli* Media

Celtone® Complete

Celtone® Complete yields a growth rate comparable to LB media, allowing for inoculation and induction within one working day. Glucose levels range from ~0.3 to 0.6 g/L, depending on the batch. Celtone® Complete is a ready-to-use medium that does not require dilution or pH adjustment. Each lot is tested for sterility, cell growth and protein expression. Celtone® Complete is available in 0.1 L and 1 L sizes.

Catalog No.	Description
CGM-1040-C	Celtone® Complete Medium (^{13}C, 98%+)
CGM-1040-D	Celtone® Complete Medium (D, 97%+)
CGM-1040-N	Celtone® Complete Medium (^{15}N, 98%+)
CGM-1040-CN	Celtone® Complete Medium (^{13}C, 98%+; ^{15}N, 98%+)
CGM-1040-DN	Celtone® Complete Medium (D, 97%+; ^{15}N, 98%+)
CGM-1040-CDN	Celtone® Complete Medium (^{13}C, 98%+; D, 97%+; ^{15}N, 98%+)
CGM-1040-U	Celtone® Complete Medium (Unlabeled)

Celtone® Powder

Celtone® Powder is CIL's most flexible nutrient-rich media. The advantage of Celtone® Powder is that researchers can formulate a custom medium based on their specific research needs. Depending on cell line and desired performance, this powdered media can be used at concentrations ranging from 1 g to 10 g per liter. Truly exceptional performance has been achieved using 10 g of Celtone® Powder in 1 L of medium containing M9 salts, 2-3 g/L glucose and 1 g of ammonium chloride (see graph on page 11). Because it is a powder, this product has the longest shelf life of any fully rich bacterial cell growth medium. Please note that if deuterium labeling is desired, D_2O must be used in media preparation. Also note that it is normal to have insoluble material present after dissolution. This material may be removed using filter paper prior to sterile filtration and will not affect performance of the medium. Celtone® Powder is available in 0.5 g and 1 g packaged sizes.

Catalog No.	Description
CGM-1030P-C	Celtone® Base Powder (^{13}C, 98%+)
CGM-1030P-D	Celtone® Base Powder (D, 97%+)
CGM-1030P-N	Celtone® Base Powder (^{15}N, 98%+)
CGM-1030P-CN	Celtone® Base Powder (^{13}C, 98%+; ^{15}N, 98%+)
CGM-1030P-DN	Celtone® Base Powder (D, 97%+; ^{15}N, 98%+)
CGM-1030P-CDN	Celtone® Base Powder (^{13}C, 98%+; D, 97%+; ^{15}N, 98%+)
CGM-1030P-U	Celtone® Base Powder (Unlabeled)

Silantes® *E. coli*-OD2

Silantes® *E. coli*-OD2 is made from bacterial hydroylsate and primarily contains amino acids, some low MW oligopeptides and almost no carbohydrates (~0.1 g of glucose/L). The bacterial strain used is a chemolithoautotrophic organism which grows on inorganic substrates that are isotopically labeled. Silantes® OD2 media are ready-to-use sterile solutions and can be used instantly for the fermentation of bacteria. Silantes® *E. coli*-OD2 media is available in 0.2 L and 1 L sizes.

Catalog No.	Description
CGM-1020-SL-C	*E. coli*-OD2 (Rich Growth Media for *E. coli*) (^{13}C, 98%)
CGM-1020-SL-D	*E. coli*-OD2 (Rich Growth Media for *E. coli*) (D, 98%)
CGM-1020-SL-N	*E. coli*-OD2 (Rich Growth Media for *E. coli*) (^{15}N, 98%)
CGM-1020-SL-CN	*E. coli*-OD2 (Rich Growth Media for *E. coli*) (^{13}C, 98%; ^{15}N, 98%)
CGM-1020-SL-DN	*E. coli*-OD2 (Rich Growth Media for *E. coli*) (D, 98%; ^{15}N 98%)
CGM-1020-SL-CDN	*E. coli*-OD2 (Rich Growth Media for *E. coli*) (^{13}C, 98%; D, 98%; ^{15}N, 98%)
CGM-1020-SL-U	*E. coli*-OD2 (Rich Growth Media for *E. coli*) (Unlabeled)

Spectra 9 Media

Spectra 9 Media is not a fully rich medium, however, it represents a cost-effective medium for *E. coli* growth and protein expression. It is comprised of labeled salts, labeled carbohydrates (>2 g glucose/L), and is supplemented with Celtone® Powder at a concentration of 1 g/L.

Catalog No.	Description
CGM-3030-C	Spectra 9 (^{13}C, 98%)
CGM-3030-D	Spectra 9 (D, 97%+)
CGM-3030-N	Spectra 9 (^{15}N, 98%)
CGM-3030-CN	Spectra 9 (^{13}C, 98%; ^{15}N, 98%)
CGM-3030-DN	Spectra 9 (D, 97%+; ^{15}N, 98%+)
CGM-3030-CDN	Spectra 9 (^{13}C, 98%; D, 97%+; ^{15}N, 98%)
CGM-3030-U	Spectra 9 (Unlabeled)

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Rich *E. coli* Media

Recommended testing protocol for BioExpress® 1000, Celtone® Media and Spectra 9

It is not necessary to add glucose or other nutrients to achieve maximal growth. All necessary substrates for optimal growth and protein expression are provided in the media. Since bacterial cell growth and recombinant protein expression can be strain specific, this is a general protocol which may need to be modified depending on the specific bacterial strain used.

Experiment 1: Growth Curve Study

Day 1

1. Beginning with a freshly grown agar plate, choose an isolated colony of bacteria and inoculate a 2 mL culture of LB (or other standard media) containing the appropriate antibiotic.
2. Shake (or rotate in a roller drum) the culture tube overnight at the appropriate temperature.

Day 2

1. In the early morning, the 2 mL culture should be dense ($OD_{600} \geq 4$). Inoculate a control media (*e.g.* LB) and the labeled media (both containing the appropriate antibiotic) with a 1:100 dilution of the overnight culture.
2. Shake (or rotate) the cultures at the appropriate temperature and collect OD_{600} data at the following time points: (T_{hours}): T_0, T_2, T_3, T_4, T_5, T_6, T_7, T_8, and approximately T_{24} (or overnight).

Experiment 2: Expression Test

Day 1

1. Beginning with a freshly grown agar plate, choose an isolated colony of bacteria and inoculate a 2 mL culture of LB (or other standard media) containing the appropriate antibiotic.
2. Shake (or rotate in a roller drum) the culture tube overnight at the appropriate temperature using identical conditions as were used for the Growth Curve study.

Day 2

1. In the early morning check the OD_{600} of the 2 mL culture (should be ≥ 4). Inoculate a control media (*i.e.* LB) and the labeled media (both containing the appropriate antibiotic) with a 1:100 dilution of the overnight culture. Depending on the number of induction time points to be analyzed, a small shake flask may be required (instead of a test tube) to grow a large enough expression culture.
2. For rich growth media induce expression of the recombinant protein when the culture reaches an OD_{600} of 0.6 to 0.8 (I_0). At the time of induction remove a 0.5 mL (or 1.0 mL) sample, spin down the culture and after carefully removing the supernatant freeze the cell pellet at -20°C.
3. Remove and process additional culture samples at the following time points (I_{hours}): I_2, I_4, I_6, I_{24}.

These cell pellets should then be used for SDS PAGE gel analysis or enzyme activity assays to compare protein expression and determine the optimum time of induction for the specific recombinant protein being produced.

Helpful Hints:

1. In general, good aeration is vital to optimal growth and expression. This requires the use of larger (18-25 mm diameter) culture tubes or small shake flasks (with baffles, if available) to allow for maximum aeration. Culture volume should be kept to 20-25% of flask volume.
2. Use freshly streaked agar plates to inoculate the growth and expression cultures.
3. Cells grow much slower in deuterated media and may require longer induction times to achieve maximum expression.

Yeast Media and Reagents

The over-expression of protein in yeast cells represents a powerful expression system for the source of properly folded and functional eukaryotic protein. Please see CIL Application Note 26 (page 71) for additional information regarding yeast as a viable expression system to produce isotope-enriched protein for NMR investigations.

Silantes® rich Yeast-OD2 Media for yeast are ready-to-use formulations. The problem of optimizing standard minimal media containing glucose or methanol as a sole carbon source therefore does not arise when using Yeast-OD2 growth media. For yeast that grows well in minimal media (*e.g. pichia*), CIL offers three different single- and double-labeled carbon sources in ^{15}N ammonium salts. Silantes® rich Yeast-OD2 Media is available in a 1 L packaged size. Please see CIL Application Note 26 (page 71) for additional information regarding the use of methanol, glucose, and ammonium salts for use in yeast.

Yeast Media and Reagents

Catalog No.	Description
CGM-4020-SL-C	Yeast-OD2 Growth Media (^{13}C, 98%)
CGM-4020-SL-N	Yeast-OD2 Growth Media (^{15}N, 98%)
CGM-4020-SL-CN	Yeast-OD2 Growth Media (^{13}C, 98%; ^{15}N, 98%)
CGM-4020-SL-U-S	Yeast-OD2 Growth Media (Unlabeled)
NLM-467	Ammonium chloride (^{15}N, 99%)
NLM-713	Ammonium sulfate ($^{15}N_2$, 99%)
CLM-1396	D-Glucose ($^{13}C_6$, 99%)
DLM-2062	D-Glucose (U-D_7, 98)
CDLM-3813	D-Glucose (U-$^{13}C_6$, 99%; U-D_7, 98%)
CLM-1510	Glycerol ($^{13}C_3$, 99%)
DLM-558	Glycerol (U-D_8, 99%)
CDLM-7745	Glycerol (U-$^{13}C_3$, 99%; U-D_8, 98%)
CLM-359	Methanol (^{13}C, 99%)
CDLM-1035	Methanol (^{13}C, 99%; D_3,98%)


Pichia pastoris as a Eukaryotic Protein Isotope-Labeling System

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The development of new systems for recombinant expression of isotopically labeled proteins is of significant interest to NMR spectroscopists. While many prokaryotic proteins can readily be over-expressed in *E. coli* and yield functional samples, over-expression of eukaryotic, and, more specifically, medically important mammalian proteins, often requires a different, non-prokaryotic, host.[1-3] Yeast is an alternative choice of eukaryotic expression system that has attracted considerable attention in recent years. Isotopic labeling of proteins in yeast for solution NMR studies began about 15 years ago.[4] Yeast is currently a second most popular expression system after *E. coli*, and seems to offer the best of the two worlds. These systems have the distinct advantages of low labeling costs, high expression yields, ease of genetic manipulation, ability to grow on deuterated media, and speeds of expression close to those in *E. coli*. At the same time, yeast expression allows native folding and an array of post-translational modifications, such as proteolytic truncation, formation of disulfide bonds, glycosylation, phosphorylation, and acylation, typical for eukaryotic cell cultures. Finally, the ability for secreted expression avoids problems with toxicity of the expressed proteins.[1]

Among several yeast species used for isotope labeling of proteins, the methylotrophic yeast *Pichia pastoris* accounts for the lion's share of produced structures (about 40 unique proteins).[5] Expression in *Pichia* usually results in higher expression yields (especially in fermenters) and more native patterns of glycosylation than in other yeast species.[6] Thus, *Pichia pastoris* is the yeast expression system of choice for today's NMR studies of eukaryotic proteins. Another popular choice, *Saccharomyces cerevisiae*, comes a distant second. So far, it has yielded nine unique protein structures, with no structures published in the last eight years.[5] Yet another yeast species, *Klyuveromyces lactis*, was recently evaluated for labeled protein expression with promising results.[1]

The uniform $^{13}C/^{15}N$ isotopic labeling in *Pichia pastoris* is very straightforward and follows established protocols, both for shaker incubators and fermenters.[6-8] A large variety of strains and vectors designed for both secreted and non-secreted expression is available from Invitrogen.[9] Among the highlights are the availability of protease-deficient strains, a number of vectors designed for efficient protein secretion (or membrane-

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Yeast Media and Reagents

Pichia pastoris as a Eukaryotic Protein Isotope-Labeling System

targeting), protein tagging, and selection for multi-copy integration transformants. The protocols for the removal of covalently and non-covalently bound sugars are available as well.[10] *Pichia pastoris* grows well on minimal media, where ^{15}N can be supplied in the form of ammonium salts, and two different ^{13}C sources are employed in the pre-induction and the post-induction phase. As protein expression is induced by methanol and is conducted under the strong alcohol oxidase promoter, ^{13}C-labeled methanol is the main carbon source in the protein expression phase. Prior to the induction, ^{13}C-labeled glycerol or glucose should be employed.[6] Economical protocols to minimize the use of those labeled precursors have been developed.[8] Yields of hundreds of milligrams of soluble secreted proteins can be achieved in fermentors at extremely high cell densities. Even flask cultures can produce tens of milligrams of doubly isotopically labeled proteins per liter of culture.[7] Important for NMR applications, a number of deuteration protocols is available, both for efficient backbone deuteration using protonated carbon sources in D_2O, as well as for full deuteration.[11,12] Finally, procedures for isotopic labeling of selected amino acid types (Cys, Leu, Lys, and Met) have been published, and Pichia strains for labeling aromatic sidechains have been developed.[13,14]

A number of interesting structures of proteins expressed in Pichia pastoris have been solved by solution NMR spectroscopy in the last 15 years, including the Man-6-P receptor diester recognition domain, cofactor-active fragment of thrombomodulin, collagen-binding domain of discoidin domain receptor 2, and many others.[5] One can expect that in the near future yeast expression systems will emerge as a very valuable tool in the NMR studies of eukaryotic membrane proteins, which are extremely hard to express functionally in bacterial systems and usually too expensive to produce in cell cultures. Multiple successful trials of functional expression of G-protein coupled receptors (GPCRs) and other membrane proteins in *Pichia pastoris* on a milligram scale[15-17] give us optimism on the future of yeast both for solution and solid-state NMR spectroscopy.

References

1. Takahashi, H.; Shimada, I. **2010**. Production of isotopically labeled heterologous proteins in non-*E. coli* prokaryotic and eukaryotic cells. *J Biomol NMR, 46*, 3-10.
2. Demain, A.L.; Vaishnav, P. **2009**. Production of recombinant proteins by microbes and higher organisms. *Biotechnol Adv, 27*, 297-306.
3. Lundstrom, K.; Wagner, R.; Reinhart, C.; Desmyter, A.; Cherouati, N.; Magnin, T.; Zeder-Lutz, G.; Courtot, M.; Prual, C.; André, N.; Hassaine, G.; Michel, H.; Cambillau, C.; Pattus, F. **2006**. Structural genomics on membrane proteins: comparison of more than 100 GPCRs in 3 expression systems. *J Struct Funct Genomics, 7*, 77-91.
4. Laroche, Y.; Storme, V.; De Meutter, J.; Messens, J.; Lauwereys, M. **1994**. High-level secretion and very efficient isotopic labeling of tick anticoagulant peptide (TAP) expressed in the methylotrophic yeast, *Pichia pastoris*. *Biotechnology (NY), 12*, 1119-24.
5. www.rcsb.org
6. Pickford, A.R.; O'Leary, J.M. **2004**. Isotopic labeling of recombinant proteins from the methylotrophic yeast *Pichia pastoris. Methods Mol Biol, 278*, 17-33.
7. Wood, M.J.; Komives, E.A. **1999**. Production of large quantities of isotopically labeled protein in *Pichia pastoris* by fermentation. *J Biomol NMR, 13*, 149-59.
8. Rodriguez, E.; Krishna, N.R. 2001. An economical method for $^{15}N/^{13}C$ isotopic labeling of proteins expressed in *Pichia pastoris. J Biochem, 130*, 19-22.
9. www.invitrogen.com
10. O'Leary, J.M.; Radcliffe, C.M.; Willis, A.C.; Dwek, R.A.; Rudd, P.M.; Downing, A.K. **2004**. Identification and removal of O-linked and non-covalently linked sugars from recombinant protein produced using *Pichia pastoris. Protein Expr Purif, 38*, 217-27.
11. Morgan, W.D.; Kragt, A.; Feeney, J. **2000**. Expression of deuterium-isotope-labeled protein in the yeast *pichia pastoris* for NMR studies. *J Biomol NMR, 17*, 337-47.
12. Massou, S.; Puech, V.; Talmont, F.; Demange, P.; Lindley, N.D.; Tropis, M.; Milon, A. **1999**. Heterologous expression of a deuterated membrane-integrated receptor and partial deuteration in methylotrophic yeasts. *J Biomol NMR, 14*, 231-9.
13. Chen, C.Y.; Cheng, C.H.; Chen, Y.C.; Lee, J.C.; Chou, S.H.; Huang, W.; Chuang, W.J. **2006**. Preparation of amino-acid-type selective isotope labeling of protein expressed in *Pichia pastoris. Proteins, 62*, 279-87.
14. Whittaker, M.M.; Whittaker, J.W. **2005**. Construction and characterization of Pichia pastoris strains for labeling aromatic amino acids in recombinant proteins. *Protein Expr Purif, 41*, 266-74.
15. Singh, S.; Hedley, D.; Kara, E.; Gras, A.; Iwata, S.; Ruprecht, J.; Strange, P.G.; Byrne, B. **2010**. A purified C-terminally truncated human adenosine A(2A) receptor construct is functionally stable and degradation resistant. *Protein Expr Purif, 74*, 80-87.
16. Sarramegna, V.; Muller, I.; Mousseau, G.; Froment, C.; Monsarrat, B.; Milon, A.; Talmont, F. **2005**. Solubilization, purification, and mass spectrometry analysis of the human mu-opioid receptor expressed in *Pichia pastoris. Protein Expr Purif, 43*, 85-93.
17. André, N.; Cherouati, N.; Prual, C.; Steffan, T.; Zeder-Lutz, G.; Magnin, T.; Pattus, F.; Michel, H.; Wagner, R.; Reinhart, C. **2006**. Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen. *Protein Sci, 15*, 1115-26.

Insect Cell Media

The Baculovirus Expression Vector System (BEVS), first introduced in the mid-1980's, has grown to become the most versatile and widely used eukaryotic vector system employed for the expression of recombinant proteins in cultured insect cells. The BEVS is based on the infection of insect cells with recombinant baculovirus (BV) carrying the gene of interest with the subsequent expression of the corresponding recombinant protein by the insect cells. The most popular insect cell lines used in conjunction with the BEVS are Sf9 (*Spodoptera frugiperda*) and High Five™ (*Trichopulsia ni*).

CIL is proud to offer BioExpress® 2000, a rich growth media for culturing insect cells. The use of BioExpress® 2000 in the uniform ^{15}N and selective amino acid-type labeling of the catalytic domain of Abl kinase is described in CIL Application Note 14 (page 47). The use of Experimental Design in the optimization of protein yield using BioExpress® 2000 is exemplified in CIL Application Note 20 (page 59).

BioExpress® 2000 is packaged as two components: a solid powder (a proprietary blend of inorganic salts, carbohydrates, and labeled amino acids) and a liquid component (fatty acid solution). Selective amino acid-type labeling is possible with BioExpress® 2000 because the amino acid content is chemically defined.

"My research group develops solid-state NMR methods for protein structure determination, requiring large quantities of ^{13}C,^{15}N-labeled samples produced in-house. We have been very pleased with CIL products for isotopically labeled protein expression, including glucose, glycerol, ammonium chloride, BioExpress®, and specifically labeled amino acids. Using CIL products, we routinely obtain several milligrams of labeled membrane proteins per liter of *E. coli* and tens to hundreds of milligrams of water-soluble proteins. We especially appreciate the competitive pricing, outstanding quality control, friendly customer service and timely delivery of a wide range of products in stock that CIL provides. It's reassuring to know that CIL values its customers and never compromises product quality."

Dr. Chad Rienstra
Chemistry Department
University of Illinois

Insect Cell Media

Catalog No.	Description
CGM-2000-N	BioExpress® 2000 (U-^{15}N, 98%)
CGM-2000-N-S	BioExpress® 2000 (U-^{15}N, 98%)
CGM-2000-CN	BioExpress® 2000 (U-^{13}C, 98%; U-^{15}N, 98%)
CGM-2000-U	BioExpress® 2000 (Unlabeled)
CGM-2000-U-S	BioExpress® 2000 (Unlabeled)
CGM-2000-CUSTOM	BioExpress® 2000 (Labeled amino acids to be specified by customer at time of request)

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

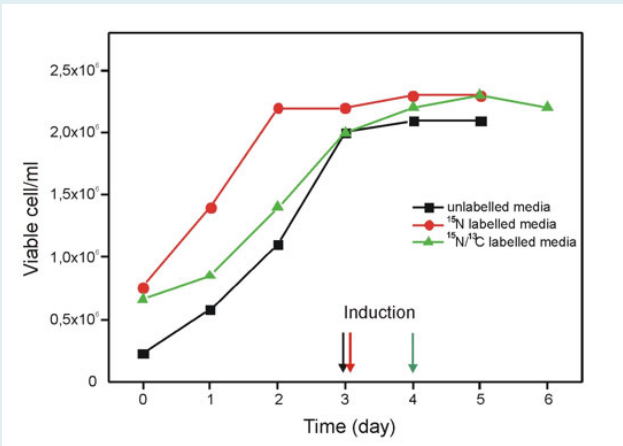
1. Prior to performing isotope labeling of a protein, optimize culture and BV-infection conditions in unlabeled labeling medium (e.g. BioExpress® 2000-U) for expression of the protein.
2. Several 100 mL cultures of Sf9 cells adapted to growth in serum-free medium SF900 II in 500 mL Erlenmeyer flasks 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 ~ 1.5 × 10^6 c/mL(~ 3 days), sterile centrifuge the cells at 400 G for 20 minutes at 20°C.
5. Resuspend 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 × 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 3 days in labeled medium post infection at 27°C, shaken at 90 rpm.
8. Harvest the cells expressing the labeled recombinant protein by centrifugation (400 G, 20 minutes. at 20°C); resuspend 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 purifiy the recombinant protein according to protocols generated for the unlabeled protein. MS and NMR analysis are carried out for proteins labeled in *E. coli*.

For more information, see Application Note 14 on page 47.

Mammalian Cell Media

There is continued interest in obtaining labeled recombinant protein from mammalian cells because eukaryotic protein expressed in mammalian cells has the greatest probability of being properly folded and functional.

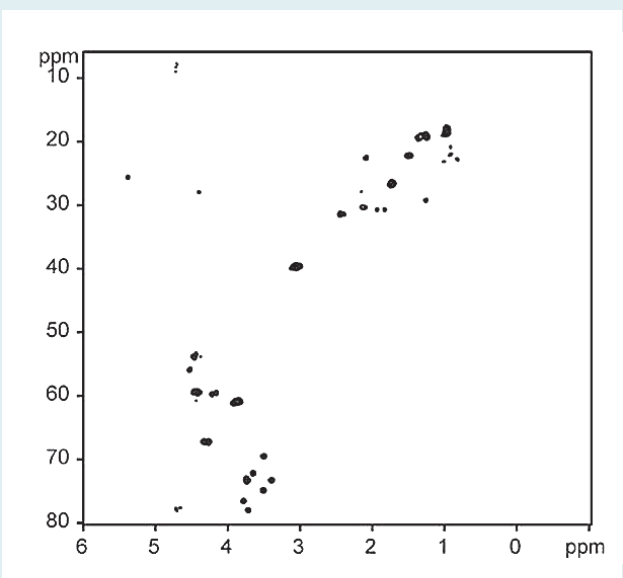
CIL offers the only commercially available labeled mammalian media intended for the production of labeled protein with yields suitable for NMR studies. Similar growth characteristics should be obtained using BioExpress® 6000 as with using Dulbecco's Modified Eagle Medium (DMEM). The amino acid content in BioExpress® 6000 is chemically defined so many different custom labeling strategies may be realized. For example, Harold Schwalbe, Karla Werner, and Judith Klein-Seetharaman at Goethe University have used BioExpress® 6000 to express rhodopsin from HEK293 cells that is labeled at the Gly, Lys, Leu, Gln, Ser, Thr, Val and Trp residues with either ^{15}N or $^{13}C,^{15}N$. Please see the presented data regarding their application, as well as a growth curve for unlabeled, ^{15}N-labeled and $^{13}C,^{15}N$-labeled BioExpress® 6000.



This graph shows the number of viable cells per mL of culture for differently labeled CIL media. Cells are induced on day three and four and harvested two days later. No differences in cell densities are seen so far. Protein yield is approximately 2.2 mg/L cell culture in all cases.

Mammalian Cell Media

Catalog No.	Description
CGM-6000-N	BioExpress® 6000 (U-^{15}N, 98%)
CGM-6000-N-S	BioExpress® 6000 (U-^{15}N, 98%)
CGM-6000-CN	BioExpress® 6000 (U-^{13}C, 98%; U-^{15}N, 98%)
CGM-6000-U-S	BioExpress® 6000 (Unlabeled)
CGM-6000-CUSTOM	BioExpress® 6000 (Labeled amino acids to be specified by customer at time of request)



^{13}C HSQC spectrum of $^{13}C,^{15}N$-labeled rhodopsin showing the sharper peaks due to the more flexible region of the spectrum.

Visit www.isotope.com for a complete listing of packaged sizes and list prices.

Cell-Free Protein Expression

CIL offers a wide variety of products for cell-free protein expression. Cell-free protein expression methods offer several advantages over expression using *E. coli* or other *in vivo* expression systems. These advantages include increased speed, ability to express toxic proteins, ease of amino acid type selective labeling, and an open system that allows cofactors, chaperones, redox molecules, and detergents to be easily be added to the expression system. Cell-free methods also allow co-expression of multiple proteins and are amenable to automation.

CIL is proud to distribute a wide range of products from CellFree Sciences (CFS) as well as Large-Scale EasyXpress™ NMR Uniform Labeling Kits manufactured by QIAGEN.® CIL also offers algal-derived amino acid mixes and conveniently packaged sizes of individual crystalline amino acids.

Amino Acid Mixes for Cell-Free Protein Expression

Catalog No.	Description
CLM-1548	Algal amino acid mixture (16AA) (U-^{13}C, 97-99%)
DLM-2082	Algal amino acid mixture (16AA) (U-D, 98%)
NLM-2161	Algal amino acid mixture (16AA) (U-^{15}N, 98%)
CNLM-452	Algal amino acid mixture (16AA) (U-^{13}C, 97-99%; U-^{15}N, 97-99%)
DNLM-819	Algal amino acid mixture (16AA) (U-D, 98%; U-^{15}N, 98%)
CDNLM-2496	Algal amino acid mixture (16AA) (U-^{13}C, 97-99%; U-D, 97-99%; U-^{15}N, 97-99%)
ULM-2314	Algal amino acid mixture (16AA) (Unlabeled)
DLM-6819	"Cell Free" amino acid mix (20AA) (U-D, 98%)
NLM-6695	"Cell Free" amino acid mix (20AA) (U-^{15}N, 96-98%)
CNLM-6696	"Cell Free" amino acid mix (20AA) (U-^{13}C, 97-99%+; U-^{15}N, 97-99%)
DNLM-6818	"Cell Free" amino acid mix (20AA) (U-D, 98%; U-^{15}N, 98%)
CDNLM-6784	"Cell Free" amino acid mix (20AA) (U-^{13}C, 97-99%; U-^{15}N, 97-99%; U-D, 97-99%)
ULM-7891	"Cell Free" amino acid mix (20AA) (Unlabeled)

QIAGEN® is a registered trademark of the QIAGEN Group.
EasyExpress™ is a registered trademark of the QIAGEN Group.

"We have been using CIL products for years and appreciate the competitive pricing, outstanding quality control, friendly customer service and timely delivery of the wide range of products that CIL provides for our protein folding and structural studies."

Dr. Lila Gierasch
Department of Biochemistry and Molecular Biology
University of Massachusetts Amherst

Profiles for 16 Amino Acid Mixture (16 AA)		Profiles for 20 Amino Acid Mixture (20 AA)	
Approximate percentages, subject to lot-to-lot variability.			
L-Alanine	7%	L-Alanine	6%
L-Arginine	7%	L-Arginine	6%
L-Aspartic acid	10%	L-Asparagine	5%
L-Glutamic acid	10%	L-Aspartic acid	8%
Glycine	6%	L-Cysteine	3%
L-Histidine	2%	L-Glutamic acid	9%
L-Isoleucine	4%	L-Glutamine	5%
L-Leucine	10%	Glycine	5%
L-Lysine	14%	L-Histidine	1%
L-Methionine	1%	L-Isoleucine	3%
L-Phenylalanine	4%	L-Leucine	9%
L-Proline	7%	L-Lysine	12%
L-Serine	4%	L-Methionine	1%
L-Threonine	5%	L-Phenylalanine	4%
L-Tyrosine	4%	L-Proline	5%
L-Valine	5%	L-Serine	4%
		L-Threonine	4%
		L-Tryptophan	3%
		L-Tyrosine	3%
		L-Valine	4%

New!

25 mg and 50 mg packaged sizes of any CIL amino acid! Experience the convenience and flexibility of using individually packaged, crystalline amino acids with your cell-free protein expression system.* See page 25 for a complete listing of CIL's free amino acids.

*CIL does not provide protocols in formulation of amino acid mixtures, as the formulations may vary depending on application and reaction scale. For first-time amino acid formulations, the pH should be checked prior to use.

Visit www.isotope.com for a complete listing of packaged sizes and list prices.

Cell-Free Protein Expression

Cell-Free Production of Stable Isotope-Labeled Proteins

Dr. Masatsune Kainosho
Tokyo Metropolitan University, Hachioji, Tokyo and Nagoya University, Nagoya, Japan

Nuclear magnetic resonance (NMR) spectroscopy is used for various purposes in protein science, such as structural biology and drug development. During the last decade, many of the long-standing methodological difficulties of protein NMR spectroscopy, such as molecular size and sensitivity limitations, have been successfully addressed. For example, using the SAIL (stereo-array isotope labeling) method, it is now routinely possible to determine the high-quality structures of proteins as large as 40-50 kDa, as easily as small proteins.[1] These technological breakthroughs emerged by the synergic development of spectroscopic methodologies and preparative methods of protein samples optimized for collecting the necessary NMR parameters in efficient and accurate manners.

A crucial issue was the development of appropriate protein expression systems, to enable NMR spectroscopists to prepare isotope-labeled protein samples in their own laboratories. It is especially important when using sophisticated stable isotope aided NMR approaches, such as the SAIL method, to choose a method that efficiently incorporates the expensive labeled amino acids into the targeted proteins without serious metabolic scrambling. This is where the cell-free protein expression systems have made a major contribution. The cell-free expression systems utilize the extracts of various living cells, which contain all of the cellular components relevant for protein synthesis. One can choose the most appropriate host cells, which can be micro-organism, plant or mammalian cells, depending on the type of protein one needs to express.

The cell-free protein expression system actually has a long history, dating back to the initial successful trial to express a protein *in vitro* using an *E. coli* cell extract in the 1960's, as described in the literature.[2] In those early days, the protein production stopped soon after the expression got started, and therefore it was not possible to prepare the amounts of protein that are required for an NMR or X-ray structural analysis. During the last few decades, however, the expression level has been enormously improved by several key modifications.[3] Now, cell-free protein synthesis methods are commonly used as important alternatives to cellular protein expression systems, for a wide variety of functional and structural studies.[4,5,6] Although most of the current NMR investigations employing cell-free protein production utilize the *E. coli* cell-free extract, it should be noted that the wheat germ extract seems to be quite useful to express "difficult" proteins, such as mammalian proteins or large protein complexes.[7] In addition to the methods using cell-extracts, there is a completely different approach that employs a reconstituted protein expression system: the "PURE system." Since the PURE system is exclusively composed of the purified components necessary for protein synthesis, it will open various new possibilities that cannot be realized by any other methods.[8]

References

1. Kainosho, M.; Torizawa, T.; Iwashita, Y.; Terauchi, T.; Ono, A.M.; Güntert, P. **2006**. Optimal isotope labeling for NMR protein structure determinations. *Nature, 440*, 52–57.
2. Kramer, G.; Kudlicki, W.; Hardesty, B. **1999**. Cell-free Coupled Transcription-Translation Systems from *Escherichia coli*. Oxford University Press, New York, pp. 129-165.
3. Spirin, A.S.; Baranov, V.I.; Ryabova, L.A.; Ovodov, S.Y.; Alakhov, Y.B. **1988**. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science, 242*, 1162-1164.
4. Kigawa, T.; Yabuki, T.; Yoshida, Y.; Tsutsui, M.; Ito, Y.; Shibata, T.; Yokoyama, S. **1999**. Cell-free production and stable-isotope labeling of milligram quantities of proteins. *FEBS Lett, 442*, 15-19.
5. Torizawa, T.; Shimizu, M.; Taoka, M.; Miyano, H.; Kainosho, M. **2004**. Efficient production of isotopically labeled proteins by cell-free synthesis: A practical protocol. *J Biomol NMR, 30*, 311–325.
6. Takeda, M.; Ikeya, T.; Güntert, P.; Kainosho, M. **2007**. Automated structure determination of proteins with the SAIL-FLYA method. *Nature Protocols, 2*, 2896-2902.
7. Madin, K.; Sawasaki, T.; Ogasawara, T.; Endo, Y. **2000**. A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc Natl Acad Sci USA, 97*, 559-564.
8. Shimizu, Y.; Inouse, A.; Tomari, Y.; Suzuki, T.; Yokogawa, T.; Nishikawa, K.; Ueda, T. **2001**. Cell-free translation reconstituted with purified components. *Nat Biotechnol, 19*, 751-755.

Cell-Free Protein Expression

CellFree Sciences (CFS) Products and Kits for Cell-Free Protein Expression

Producing proteins at will, often a bottleneck in post-genome studies, has become a reality with the advent of the robust wheat germ cell-free protein expression system. CellFree Sciences' ENDEXT® wheat germ cell-free system permits both high throughput protein screening and microgram- to milligram-scale protein production overnight. Protein synthesis protocols for the ENDEXT® system have been optimized for a wide range of proteins. They have also been incorporated into robotic protein synthesizers, versatile Protemist® DT II and mass-producing Protemist® XE. Being eukaryotic and free from physiological constraints that hamper *in vivo* systems, the wheat germ cell-free system allows synthesis, with or without additives, of a broad spectrum of protein and protein complexes ranging from 10 kDa to 360 kDa in well-folded and soluble forms. Most importantly, labeled protein samples can be easily produced for NMR studies when labeled amino acids are used.

Please contact CFS directly (tech-sales@cfsciences.com) if you would like to use CFS's lab services to prepare a pEU plasmid with your target gene sequence, characterize the yield and solubility of your expressed protein, or produce a prescribed amount of protein using the wheat germ cell-free system.

CFS Product Listing

Standard Reagents and Kits

Catalog #	Short Description	Description
Transcription Reagents		
CFS-TSC-5TB	5x Transcription Buffer	Transcription buffer for WEPRO1240/2240 series
CFS-TSC-ENZ	SP6 RNA Polymerase + RNase Inhibitor	Enzymes for mRNA synthesis and RNase activity inhibition
CFS-TSC-NTP	NTP Mix	Mixture of high quality substrates for transcription
Translation Reagents		
CFS-WGE-1240	WEPRO1240	WGE (240 OD) for general purpose
CFS-WGE-1240G	WEPRO1240G	WGE (240 OD) to produce high purity GST-tagged proteins
CFS-WGE-1240H	WEPRO1240H	WGE (240 OD) to produce high purity His-tagged proteins
CFS-WGE-2240	WEPRO2240	WGE (240 OD) to produce labeled proteins; WEPRO1240 less amino acids
CFS-WGE-2240H	WEPRO2240H	WGE (240 OD) to produce His-tagged, labeled proteins; WEPRO1240H less amino acids
CFS-SUB-AMX	SUB-AMIX	Buffer containing ATP, GTP, and 20 amino acids; used with WEPRO1240 series
CFS-SUB-NAA	SUB-AMIX NA	Buffer containing no amino acids; used with WEPRO2240 series
CFS-SUB-AMX-N	SUB-AMIX (^{15}N, 97-99%)	Buffer containing ^{15}N-labeled amino acids; used with WEPRO2240 series
CFS-SUB-AMX-CN	SUB-AMIX (^{13}C, 97-99%; ^{15}N, 97-99%)	Buffer containing ^{13}C,^{15}N-labeled amino acids; used with WEPRO2240 series
CFS-SUB-AMX-CDN	SUB-AMIX (^{13}C, 97-99%; D, 97-99%; ^{15}N, 97-99%)	Buffer containing ^{13}C,D,^{15}N-labeled amino acids; used with WEPRO2240 series
Small Scale Expression Kits		
CFS-EDX-S	Premium Expression Kit	Trial expression kit using plasmid template for six (6) 227-µl reactions
CFS-EDX-PCR	Premium Expression Kit, PCR	Trial expression kit using PCR template for six (6) 227-µl reactions
Large Scale Expression Kits		
CFS-TRI-1240	WEPRO1240 Expression Kit	Kit containing 2 mL WEPRO1240
CFS-TRI-1240G	WEPRO1240G Expression Kit	Kit containing 2 mL WEPRO1240G
CFS-TRI-1240H	WEPRO1240H Expression Kit	Kit containing 2 mL WEPRO1240H
CFS-TRI-2240H	WEPRO2240H Expression Kit	Kit containing 2 mL WEPRO2240H and SUB-AMIX NA
CFS-TRI-2240-N	WEPRO2240 (^{15}N) Expression Kit	Kit containing 2 mL WEPRO2240 and ^{15}N-labeled amino acids
CFS-TRI-2240-CN	WEPRO2240 (^{13}C,^{15}N) Expression Kit	Kit containing 2 mL WEPRO2240 and ^{13}C,^{15}N-labeled amino acids
CFS-TRI-2240-CDN	WEPRO2240 (^{13}C,D,^{15}N) Expression Kit	Kit containing 2 mL WEPRO2240 and ^{13}C,D,^{15}N-labeled amino acids

ENDEXT® is a registered trademark of CellFree Sciences.
Protemist® is a registered trademark of Emerald BioSystems.

CIL is a distributor of the above-referenced CFS products in the US and Europe.

Cell-Free Protein Expression

Catalog #	Short Description	Description
CFS-TRI-2240H-N	WEPRO2240H (^{15}N) Expression Kit	Kit containing 2 mL WEPRO2240H and ^{15}N-labeled amino acids
CFS-TRI-2240H-CN	WEPRO2240H (^{13}C, ^{15}N) Expression Kit	Kit containing 2 mL WEPRO2240H and ^{13}C,^{15}N-labeled amino acids
CFS-TRI-2240H-CDN	WEPRO2240H (^{13}C, D, ^{15}N) Expression Kit	Kit containing 2 mL WEPRO2240H and ^{13}C,D,^{15}N-labeled amino acids

High Performance Reagents and Kits

Transcription Reagents		
CFS-TSC-5TB-LM	5x Transcription Buffer LM	Transcription buffer for WEPRO7240/8240 series
CFS-TSC-ENZ	SP6 RNA Polymerase + RNase Inhibitor	Enzymes for mRNA synthesis and RNase activity inhibition
CFS-TSC-NTP	NTP Mix	Mixture of high quality substrates for transcription
Translation Reagents		
CFS-WGE-7240	WEPRO7240	High yield WGE (240 OD) for general purpose
CFS-WGE-7240G	WEPRO7240G	High yield WGE (240 OD) to produce high purity GST-tagged proteins
CFS-WGE-7240H	WEPRO7240H	High yield WGE (240 OD) to produce high purity His-tagged proteins
CFS-WGE-8240	WEPRO8240	High yield WGE (240 OD) to produce labeled proteins; WEPRO7240 less amino acids
CFS-WGE-8240H	WEPRO8240H	High yield WGE (240 OD) to produce high purity His-tagged, labeled proteins; WEPRO7240H less amino acids
CFS-SUB-SGC	SUB-AMIX SGC	Buffer for BL for general purpose; used with WEPRO7240 series
CFS-SUB-SGC-NAA	SUB-AMIX SGC NA	Buffer for BL, containing no amino acids; used with WEPRO8240 series
CFS-SUB-SG	SUB-AMIX SG	Buffer for FF for general purpose; used with WEPRO7240 series
CFS-SUB-SG-NAA	SUB-AMIX SG NA	Buffer for FF, containing no amino acids ; used with WEPRO8240 series
CFS-SUB-SGC-N	SUB-AMIX SGC (^{15}N, 97-99%)	Buffer for BL, containing ^{15}N-labeled amino acids; used with WEPRO8240 series
CFS-SUB-SGC-CN	SUB-AMIX SGC (^{13}C, 97-99%; ^{15}N, 97-99%)	Buffer for BL, containing ^{13}C,^{15}N-labeled amino acids; used with WEPRO8240 series
CFS-SUB-SGC-CDN	SUB-AMIX SGC (^{13}C, 97-99%; D, 97-99%; ^{15}N, 97-99%)	Buffer for BL, containing ^{13}C,D,^{15}N-labeled amino acids; used with WEPRO8240 series
CFS-SUB-SG-N	SUB-AMIX SG (^{15}N, 97-99%)	Buffer for FF, containing ^{15}N-labeled amino acids; used with WEPRO8240 series
CFS-SUB-SG-CN	SUB-AMIX SG (^{13}C, 97-99%; ^{15}N, 97-99%)	Buffer for FF, containing ^{13}C,^{15}N-labeled amino acids; used with WEPRO8240 series
CFS-SUB-SG-CDN	SUB-AMIX SG (^{13}C, 97-99%; D, 97-99%; ^{15}N, 97-99%)	Buffer for FF, containing ^{13}C,D,^{15}N-labeled amino acids; used with WEPRO8240 series
Large Scale Expression Kits		
CFS-TRI-7240	WEPRO7240 Expression Kit	Contains 2mL WEPRO7240
CFS-TRI-7240G	WEPRO7240G Expression Kit	Contains 2 mL WEPRO7240G
CFS-TRI-7240H	WEPRO7240H Expression Kit	Contains 2 mL WEPRO7240H
CFS-TRI-8240H-N	WEPRO8240H (^{15}N) Expression Kit	Contains 2 mL WEPRO8240H and ^{15}N-labeled amino acids
CFS-TRI-8240H-CN	WEPRO8240H (^{13}C,^{15}N) Expression Kit	Contains 2 mL WEPRO8240H and ^{13}C,^{15}N-labeled amino acids
CFS-TRI-8240H-CDN	WEPRO8240H (^{13}C,D,^{15}N) Expression Kit	Contains 2 mL WEPRO8240H and ^{13}C,D,^{15}N-labeled amino acids

WGE: Wheat germ extract BL: Bilayer method FF: Filter-and-Feed method

Visit www.isotope.com for a complete listing of packaged sizes and list prices.

(continued)

Cell-Free Protein Expression

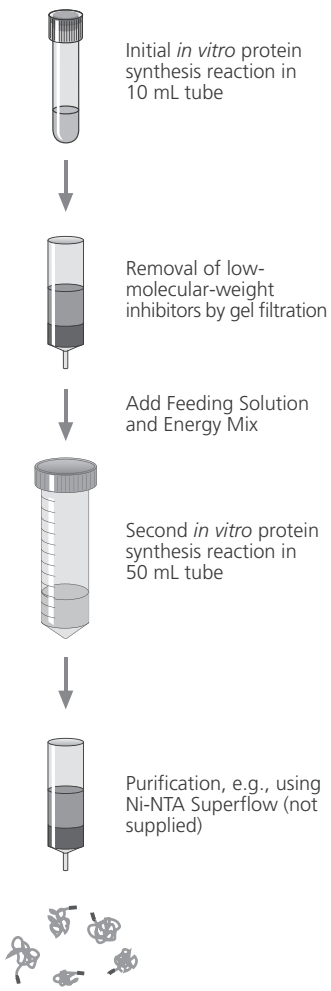
QIAGEN EasyXpress™ NMR Protein Synthesis Kits contain all equipment and reagents required to make uniform labeled protein in either 2x5 mL or 10x1 mL reactions. These kits are easy to use and can produce uniform single-, double- or triple-labeled protein in as little as 2-3 hours. The kit makes use of an *E. coli* extract that contains the molecular machinery required for efficient protein synthesis. QIAGEN EasyXpress™ kits can be used to express proteins from a variety of DNA templates, as long as they contain a T7 or other strong *E. coli* promoter (*e.g.*, T5) upstream from the coding sequence, and a ribosome binding site. Please visit www.qiagen.com/products/protein/expression/easyxpress/default.aspx for more information regarding QIAGEN's complete EasyXpress™ portfolio, which includes cloning vectors, kits for template generation, and small-scale kits for expression evaluation purposes.

Catalog No.	Description
QIA-32531-N	QIAGEN EasyXpress™ NMR Kit (U-^{15}N, 97-99%)
QIA-32532-CN	QIAGEN EasyXpress™ NMR Kit (U-^{13}C, 97-99%; U-^{15}N, 97-99%)
QIA-32535-CDN	QIAGEN EasyXpress™ NMR Kit (U-^{13}C, 97-99%; U-D, 97-99%; U-^{15}N, 97-99%)

"In our laboratory we frequently deal with backbone resonance assignments of perdeuterated proteins larger than 40 kDa. Compared to traditional *E. coli* expression systems, *in vitro* translation has the potential to simplify the assignment procedure in difficult proteins. We have been impressed by the effectiveness of the QIAGEN EasyXpress™ NMR Kit, with respect to the ease of its use and the final yield of purified protein. We expect that this technology will greatly facilitate our future scientific efforts."

Dr. Ronnie Ghose
Department of Chemistry
City College of New York
New York, NY

QIAGEN EasyXpress™ Large-Scale Procedure



Visit www.isotope.com for a complete listing of packaged sizes and list prices.

Selective Amino Acid-Type Labeling

Selective amino acid-type labeling is used to simplify crowded spectra in order to aid in spectral interpretation or to provide specific probes for dynamic and structural studies. This technique requires the addition of free labeled amino acids (~50->250 mg/L) to either minimal or rich growth media prior to protein induction. The cells will generally utilize the supplemented amino acids for protein synthesis prior to undergoing the *de novo* synthesis of the target amino acids. Please see page 25 for a complete listing of amino acids that may be used with either *in vivo* growth systems or cell-free protein expression methods.

Selective Isotope-Labeling Methods for Protein Structural Studies

Hanudatta S. Atreya, Ph.D.
NMR Research Centre, Indian Institute of Science, Bangalore, India

One of the major contributing factors to the rapid advance of biomolecular NMR spectroscopy is the emergence of different isotope labeling methods. Recent developments in biotechnology have made it easier and economical to introduce ^{13}C, ^{15}N and ^{2}H into proteins and nucleic acids. At the same time, there has been an explosion in the number of NMR experiments that utilize such isotope-labeled samples. Thus, a combination of isotopic labeling and multidimensional, multinuclear experiments has significantly expanded the range of problems in structural biology amenable to NMR.

Isotope labeling in proteins can be broadly classified into four categories: Uniform, amino acid-type selective, site-specific and random/fractional labeling. The beginning of systematized isotope labeling in proteins can be traced back to late 60's in the group of Jardetsky and Katz and coworkers.[1,2] Theirs was also one of the first amino acid-type selective labeling methods involving incorporation of specific protonated amino acids against a deuterated background. In the 80's uniform ($^{13}C/^{15}N$) and selective incorporation of ^{15}N-labeled amino acids against an unlabeled ($^{12}C/^{14}N$) background was developed.[3] Subsequently, a variety of labeling methods have emerged (reviewed in [4] and [5] and illustrated in Figure 1).

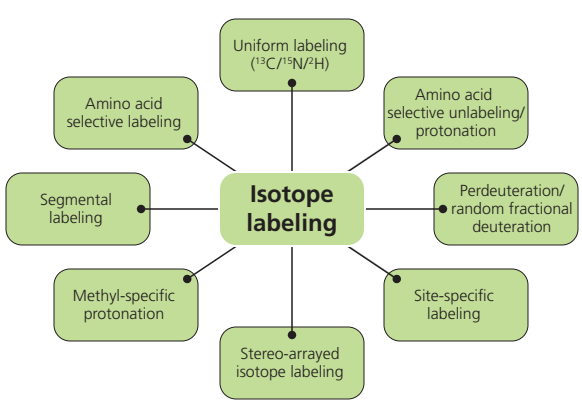


Figure 1. Different isotope-labeling methods.

In addition to uniform ($^{13}C/^{15}N/^{2}H$) labeling, amino acid-type or site-selective labeling is often pursued as it helps in spectral simplification and provides specific probes for structural and dynamic studies. Selective amino acid-type labeling also aids in sequence-specific resonance assignments by helping to identify resonances which are otherwise buried in the crowded regions of 2D and 3D NMR spectra. However, a disadvantage of this method is the possible mis-incorporation of ^{15}N label in undesired amino acids (also called as "isotope scrambling").[3] This happens due to metabolic conversion of one amino acid to another in the bio-synthetic pathway of the cell. The problem becomes more severe for amino acids higher up or intermediates in the metabolic pathway such as Asp, Glu and Gln (See Figure 2 showing the biosynthetic pathway in *E. coli*). For those which are end-products in the production pipeline (Ala, Arg, Asn, Cys, His, Ile, Lys, Met, Pro and Trp) isotope scrambling is minimal and the remaining (Gly, Phe, Leu, Ser, Thr, Tyr and Val) have medium to weak interconversion. Isotope scrambling in *E. coli* can be minimized by reducing the activity of the enzyme(s) catalyzing the inter-conversion or amino transfer using either specific (auxotrophic) strains[3] or using enzyme inhibitors.[6] Another alternative is to use cell-free or *in vitro* expression systems which lack these enzymes.[4]

One drawback of amino acid selective labeling is the expense associated with the use of $^{13}C/^{15}N$ labeled amino acids. A relatively inexpensive method is that of amino acid selective "unlabeling" or reverse labeling. In this method, the host organism is grown on a medium containing the desired unlabeled (*i.e.*, $^{1}H/^{12}C/^{14}N$) amino acid against a labeled ($^{13}C/^{15}N$) background. This is somewhat akin to the selective protonation experiment by Jardetsky[1] and Katz.[2] Reverse labeling was first used by Bax and coworkers[7] and developed further by other groups for different applications.[8,9,10] The problem of isotope scrambling (in this case being the mis-incorporation of ^{14}N) remains largely the same as in the selective-labeling approach mentioned above (for a detailed table of possible scrambling of ^{14}N see reference 10).

(continued)

Selective Amino Acid-Type Labeling

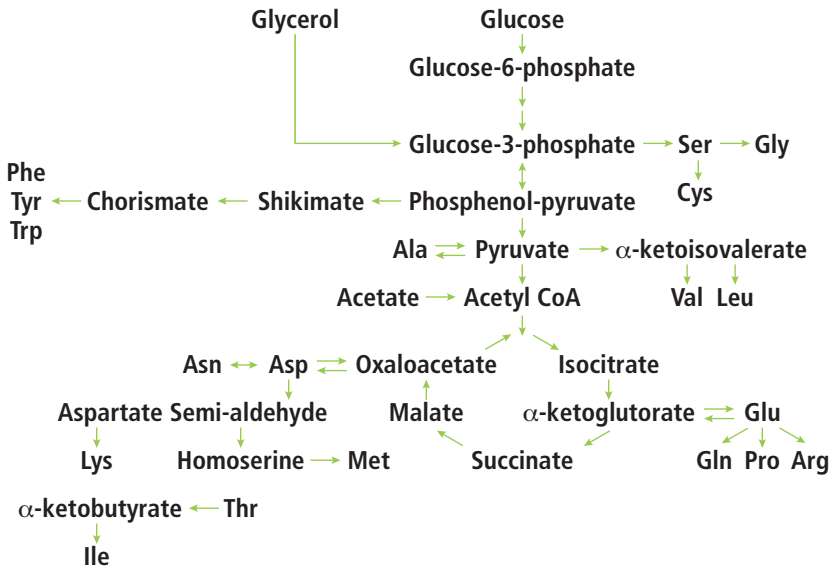


Figure 2. Amino acid biosynthesis in *E. coli.*

In addition to the above, new isotope-labeling methods continue to be developed. More recent methods of segmental labeling[11] and stereo-arrayed isotope labeling (SAIL)[12] open up new avenues in protein structural studies. The future points towards a combination of different isotope-labeling methods to address challenging and complex problems in structural biology.

References

1. Markley, J.K.; Putter, I.; and Jardetsky, O. **1968**. High-resolution nuclear magnetic resonance spectra of selectively deuterated staphylococcal nuclease. *Science, 161*,1249-1251.
2. Crespi, H.L.; Rosenberg, R.M.; and Katz, J.L. (1968) **1968**. Proton magnetic resonance of proteins fully deuterated except for H-leucine side chains. *Science, 161*,795-796.
3. Muchmore D.D.; McIntosh, L.P.; Russell, C.B.; Anderson, D.E.; Dahlquist, F.W. **1989**. Expression and nitrogen-15 labeling of proteins for proton and nitrogen-15 nuclear magnetic resonance. *Methods Enzymol, 177*, 44-73.
4. Ohki, S. and Kainosho, M. **2008**. Stable isotope labeling for protein NMR. *Prog NMR Spectrosc, 53*, 208-226.
5. *J. Biomol NMR,* **2010**. Special issue: Vol 46: 1-125.
6. Tong, K.I.; Yamamoto, M.; and Tanaka, T. **2008**. A simple method for amino acid selective isotope labeling of recombinant proteins in *E. coli. J Biomol NMR, 42*, 59-67.
7. Vuister, G.W.; Kim, S.J.; Wu, C., Bax, A. **1994**. 2D and 3D NMR study of phenylalanine residues in proteins by reverse isotopic labeling. *J Am Chem Soc, 116*, 9206-9210.
8. Shortle, D. **1995**. Assignment of amino acid type in ^{1}H-^{15}N correlation spectra by labeling with ^{14}N amino acids. *J Magn Reson B, 105*, 88-90.
9. Atreya, H.S. and Chary, K.V.R. **2001**. Selective unlabeling of amino acids in fractionally ^{13}C-labeled proteins: An approach for stereospecific NMR assignments of CH3 groups in Val and Leu residues. *J Biomol NMR, 19*, 267-272.
10. Krishnarjuna, B.; Jaiupuria, G.; Thakur, A.D.; Silva, P.; and Atreya, H.S. **2010**. Amino acid selective unlabeling for sequence specific resonance assignments in proteins. *J Biomol NMR, 49*, 38-51.
11. Cowburn, D. and Muir, T.W. **2001**. Segmental isotopic labeling for structural biological applications of NMR. *Methods Enzymol, 339*, 41–54.
12. Kainosho, M.; Torizawa, T.; Iwashita, Y.; Terauchi, T.; Ono, A.M.; and Peter Güntert. **2006**. Structure of the putative 32 kDa myrosinase binding protein from arabidopsis (At3g16450.1) determined by SAIL-NMR. *Nature, 440*, 52–57.

"We have been a loyal customer of CIL for stable isotope-labeled products for our research in the study of proteins. CIL has provided reliable, in stock, high quality products for our lab. We have enjoyed a nice working relationship with CIL over the years."

Dr. Ravi Pratap Barnwal
Research Associate, Department of Chemisty
University of Washington

Free Amino Acids

CIL offers a comprehensive list of labeled free amino acids for use in a variety of NMR investigations. The large number of available labeling schemes will maximize flexibility involved in designing experiments to probe structure, dynamics and binding of biological macromolecules. A number of packaged sizes are available, including 25 mg and 50 mg sizes, which are convenient for use in cell-free protein expression.

Free Amino Acids

Catalog No.	Description
CLM-116	L-Alanine (1-^{13}C, 99%)
CLM-2016	L-Alanine (2-^{13}C, 99%)
CLM-117	L-Alanine (3-^{13}C, 99%)
CLM-2734	L-Alanine (2,3-$^{13}C_2$, 99%)
CLM-2184	L-Alanine (U-$^{13}C_3$, 97-99%)
DLM-248	L-Alanine (3,3,3-D_3, 99%)
DLM-250	L-Alanine (2,3,3,3-D_4, 98%)
DLM-251	L-Alanine (D_7, 98%)
NLM-454	L-Alanine (^{15}N, 98%)
CDLM-8649	L-Alanine (3-^{13}C, 99%; 2-D, 96%)*
CDLM-3439	L-Alanine (3-^{13}C, 99%; 3,3,3-D_3, 98%)
CNLM-6993	L-Alanine (1-^{13}C, 99%; ^{15}N, 98%)
CNLM-3594	L-Alanine (2-^{13}C, 99%; ^{15}N, 98%)
CNLM-534	L-Alanine (U-$^{13}C_3$, 97-99%; ^{15}N, 97-99%)
DNLM-7178	L-Alanine (2,3,3,3-D_4, 98%; ^{15}N, 98%)
CDNLM-6800	L-Alanine (U-$^{13}C_3$, 97-99%; U-D_4, 97-99%; ^{15}N, 97-99%)
CLM-8755	β-Alanine (3-^{13}C, 99%)
CLM-8756	β-Alanine (1,2,3-$^{13}C_3$, 99%)
CNLM-3440	β-Alanine (3-^{13}C, 99%; ^{15}N, 98%)
CNLM-3946	β-Alanine (U-$^{13}C_3$, 98%+; ^{15}N, 96-99%)
NLM-1656	β-Alanine (^{15}N, 98%+)
CLM-2070	L-Arginine•HCL (guanido-^{13}C, 99%)
CLM-2051	L-Arginine•HCL (1,2-$^{13}C_2$, 99%)
CLM-2265-H	L-Arginine•HCL (U-$^{13}C_6$, 99%)
DLM-541	L-Arginine•HCL (D_7, 98%)
NLM-395	L-Arginine•HCL (guanido-$^{15}N_2$, 98%+)
NLM-396	L-Arginine•HCL (U-$^{15}N_4$, 98%)
CDLM-3789	L-Arginine•HCL (5-^{13}C, 99%; 4,4,5,5-D_4, 95%)
CNLM-7819	L-Arginine•HCL (1-^{13}C, 99%; α-^{15}N, 98%)
CNLM-539-H	L-Arginine•HCL (U-$^{13}C_6$, 99%; U-$^{15}N_4$, 99%)
DNLM-7543	L-Arginine•HCL (D_7, 98%; $^{15}N_4$, 98%)
CDNLM-6801	L-Arginine•HCL (U-$^{13}C_6$, 97-99%; U-D_7, 97-99%; U-$^{15}N_4$, 97-99%)
CLM-627	L-Aspartic acid (3-^{13}C, 99%)
CLM-4455	L-Aspartic acid (1,4-$^{13}C_2$, 99%)
CLM-1801	L-Aspartic acid (U-$^{13}C_4$, 97-99%)
DLM-546	L-Aspartic acid (2,3,3-D_3, 98%)
NLM-718	L-Aspartic acid (^{15}N, 98%)
CNLM-7817	L-Aspartic acid (1,4-$^{13}C_2$, 99%; ^{15}N, 98%)
CNLM-544	L-Aspartic acid (U-$^{13}C_4$, 97-99%; ^{15}N, 97-99%)
DNLM-6931	L-Aspartic acid (2,3,3-D_3, 98%; ^{15}N, 98%)
CDNLM-6803	L-Aspartic acid (U-$^{13}C_4$, 97-99%; U-D_3, 97-99%; ^{15}N, 97-99%)
CLM-3852	L-Cysteine (1-^{13}C, 99%)
CLM-1868	L-Cysteine (3-^{13}C, 99%)
CLM-4320	L-Cysteine (U-$^{13}C_3$, 97-99%)
DLM-769	L-Cysteine (3,3-D_2, 98%)
DLM-6901	L-Cysteine (2,3,3-D_3, 98%)
NLM-2295	L-Cysteine (^{15}N, 98%)
CNLM-7815	L-Cysteine (1-^{13}C, 99%; ^{15}N, 98%)
CNLM-3871	L-Cysteine (U-$^{13}C_3$, 97-99%; ^{15}N, 97-99%)
DNLM-6902	L-Cysteine (2,3,3-D_3, 98%; ^{15}N, 98%)
CDNLM-6809	L-Cysteine (U-$^{13}C_3$, 97-99%; U-D_3, 97-99%; ^{15}N, 97-99%)
CLM-520	L-Cystine (3,3'-$^{13}C_2$, 99%)
DLM-1000	DL-Cystine (3,3,3',3'-D_4, 98%)
NLM-3818	L-Cystine ($^{15}N_2$, 98%)
CDNLM-8659	L-Cystine ($^{13}C_6$, 98%; D_6, 98%; $^{15}N_2$, 98%) (CP 95%)
CLM-3612	L-Glutamine (1-^{13}C, 99%)
CLM-1166	L-Glutamine (5-^{13}C, 99%)
CLM-2001	L-Glutamine (1,2-$^{13}C_2$, 99%)
CLM-1822	L-Glutamine (U-$^{13}C_5$, 97-99%)
DLM-1826	L-Glutamine (2,3,3,4,4-D_5, 97%)
NLM-1016	L-Glutamine (α-^{15}N, 98%)
NLM-557	L-Glutamine (amide-^{15}N, 98%+)
NLM-1328	L-Glutamine ($^{15}N_2$, 98%)
CNLM-7813	L-Glutamine (1-^{13}C, 99%; α-^{15}N, 98%)
CNLM-1275	L-Glutamine (U-$^{13}C_5$, 97-99%; U-$^{15}N_2$, 97-99%)
DNLM-6997	L-Glutamine (2,3,3,4,4-D_5, 98%; $^{15}N_2$, 98%)
CDNLM-6805	L-Glutamine (U-$^{13}C_5$, 97-99%; U-D_5, 97-99%; U-$^{15}N_2$, 97-99%)
CLM-674	L-Glutamic acid (1-^{13}C, 99%)
CLM-2024	L-Glutamic acid (1,2-$^{13}C_2$, 99%)
CLM-1800	L-Glutamic acid (U-$^{13}C_5$, 97-99%)
DLM-3725	L-Glutamic acid (2,4,4-D_3, 97-98%)

*See CIL Application Note 25, page 67

(continued)

Free Amino Acids

Catalog No.	Description
DLM-556	L-Glutamic acid (2,3,3,4,4-D_5, 97-98%)
NLM-135	L-Glutamic acid (^{15}N, 98%)
CNLM-7812	L-Glutamic acid (1-^{13}C, 99%; ^{15}N, 98%)
CNLM-554	L-Glutamic acid (U-$^{13}C_5$, 97-99%; ^{15}N, 97-99%)
DNLM-6996	L-Glutamic acid (2,3,3,4,4-D_5, 98%; ^{15}N, 98%)
CDNLM-6804	L-Glutamic acid (U-$^{13}C_5$, 97-99%; U-D_5, 97-99%; ^{15}N, 97-99%)
CLM-422	Glycine (1-^{13}C, 99%)
CLM-136	Glycine (2-^{13}C, 99%)
CLM-1017	Glycine (1,2-$^{13}C_2$, 97-99%)
DLM-1674	Glycine (2,2-D_2, 98%)
DLM-280	Glycine (D_5, 98%)
NLM-202	Glycine (^{15}N, 98%)
CDLM-6072	Glycine (1-^{13}C, 99%; D_5, 98%)
CNLM-507	Glycine (1-^{13}C, 99%; ^{15}N, 98%+)
CNLM-508	Glycine (2-^{13}C, 99%; ^{15}N, 98%+)
CNLM-1673	Glycine (U-$^{13}C_2$, 97-99%; ^{15}N, 97-99%)
DNLM-6862	Glycine (2,2-D_2, 98%; ^{15}N, 98%)
CDNLM-6799	Glycine (U-$^{13}C_2$, 97-99%; 2,2-D_2, 97-99%; ^{15}N, 97-99%)
CLM-1512	L-Histidine•HCL•H_2O (<5% D) (ring-2-^{13}C, 99%)
CLM-2264	L-Histidine•HCL•H_2O (<5% D) (U-$^{13}C_6$, 97-99%)
DLM-7855	L-Histidine•HCL•H_2O (ring-2,4-D_2; α, β, β-D_3, 98%)
NLM-2245	L-Histidine•HCL•H_2O (α-^{15}N, 98%+)
NLM-846	L-Histidine•HCL•H_2O (ring-π-^{15}N, 98%+) (<5% D)
NLM-1513	L-Histidine•HCL•H_2O (<5% D) ($^{15}N_3$, 98%)
CNLM-758	L-Histidine•HCL•H_2O (<5% D) (U-$^{13}C_6$, 97-99%; U-$^{15}N_3$, 97-99%)
DNLM-7366	L-Histidine•HCL•H_2O (U-D_5, 98%; U-$^{15}N_3$, 98%)
CDNLM-6806	L-Histidine•HCL•H_2O (U-$^{13}C_6$, 97-99%; U-D_5, 97-99%; U-$^{15}N_3$, 97-99%)
CLM-1026	L-Isoleucine (1-^{13}C, 99%)
CLM-2248	L-Isoleucine (U-$^{13}C_6$, 97-99%)
DLM-141	L-Isoleucine (D_{10}, 98%)
NLM-292	L-Isoleucine (^{15}N, 98%)
CNLM-7810	L-Isoleucine (1-^{13}C, 99%; ^{15}N, 98%)
CNLM-561	L-Isoleucine (U-$^{13}C_6$, 97-99%; ^{15}N, 97-99%)
DNLM-7325	L-Isoleucine (D_{10}, 98%; ^{15}N, 98%)
CDNLM-6807	L-Isoleucine (U-$^{13}C_6$, 97-99%; U-D_{10}, 97-99%; ^{15}N, 97-99%)
CLM-468	L-Leucine (1-^{13}C, 99%)
CLM-2014	L-Leucine (2-^{13}C, 99%)
CLM-3524	L-Leucine (1,2-$^{13}C_2$, 99%)
CLM-2262	L-Leucine (U-$^{13}C_6$, 97-99%)
DLM-1259	L-Leucine (5,5,5-D_3, 98%)

Catalog No.	Description
DLM-4212	L-Leucine (isopropyl-D_7, 98%)
DLM-567	L-Leucine (D_{10}, 98%)
NLM-142	L-Leucine (^{15}N, 98%)
CNLM-615	L-Leucine (1-^{13}C, 99%; ^{15}N, 98%+)
CNLM-3450	L-Leucine (2-^{13}C, 99%; ^{15}N, 95-99%)
CNLM-281-H	L-Leucine (U-$^{13}C_6$, 99%; ^{15}N, 99%)
DNLM-4642	L-Leucine (D_{10}, 98%; ^{15}N, 97%)
CDNLM-6808	L-Leucine (U-$^{13}C_6$, 97-99%; U-D_{10}, 97-99%; ^{15}N, 97-99%)
CLM-653	L-Lysine•2HCL (1-^{13}C, 99%)
CLM-632	L-Lysine•2HCL (6-^{13}C, 99%)
CLM-2247-H	L-Lysine•2HCL (U-$^{13}C_6$, 99%)
DLM-2640	L-Lysine•2HCL (4,4,5,5-D_4, 96-98%)
DLM-570	L-Lysine•2HCL (D_9, 98%)
NLM-143	L-Lysine•2HCL (α-^{15}N, 95-99%)
NLM-631	L-Lysine•2HCL (ε-^{15}N, 98%+)
NLM-1554	L-Lysine•2HCL ($^{15}N_2$, 98%+)
CNLM-7821	L-Lysine•2HCL (1-^{13}C, 99%; α-^{15}N, 98%)
CNLM-291-H	L-Lysine•2HCL (U-$^{13}C_6$, 99%; U-$^{15}N_2$, 99%)
DNLM-7545	L-Lysine•2HCL (D_9, 98%; $^{15}N_2$, 98%)
CDNLM-6810	L-Lysine•2HCL (U-$^{13}C_6$, 97-99%; U-D_9, 97-99%; U-$^{15}N_2$, 97-99%)
CLM-893	L-Methionine (U-$^{13}C_5$,97-99%)
DLM-6797	L-Methionine (2,3,3,4,4-D_5; methyl-D_3, 98%)
NLM-752	L-Methionine (^{15}N, 96-98%)
CDLM-760	L-Methionine (1-^{13}C, 99%; methyl-D_3, 98%)
CDLM-8885	L-Methionine (2,3,3,4,4-D_5, 98%; methyl-$^{13}CH_3$, 99%)
CNLM-759	L-Methionine (U-$^{13}C_5$, 97-99%; ^{15}N, 97-99%)
DNLM-7179	L-Methionine (D_8, 98%; ^{15}N, 98%)
CDNLM-6798	L-Methionine (U-$^{13}C_5$, 97-99%; U-D_8, 97-99%; ^{15}N, 97-99%)
CLM-762	L-Phenylalanine (1-^{13}C, 99%)
CLM-1631	L-Phenylalanine (2-^{13}C, 99%) (CP 97%)
CLM-1053	L-Phenylalanine (3-^{13}C, 99%)
CLM-1055	L-Phenylalanine (ring-$^{13}C_6$, 99%)
CLM-2250	L-Phenylalanine (U-$^{13}C_9$, 97-99%)
DLM-1258	L-Phenylalanine (ring-D_5, 98%)
DLM-372	L-Phenylalanine (D_8, 98%)
NLM-108	L-Phenylalanine (^{15}N, 98%)
CNLM-7611	L-Phenylalanine (2,3-$^{13}C_2$, 99%; ^{15}N, 98%)*
CNLM-575	L-Phenylalanine (U-$^{13}C_9$, 97-99%; ^{15}N, 97-99%)
DNLM-7180	L-Phenylalanine (D_8, 98%; ^{15}N, 98%)
CDNLM-6811	L-Phenylalanine (U-$^{13}C_9$, 97-99%; U-D_8, 97-99%; ^{15}N, 97-99%)

*For solid-state NMR, see CIL Application Note 22 on page 63.

Free Amino Acids

Catalog No.	Description
CLM-510	L-Proline (1-^{13}C, 99%)
CLM-2260	L-Proline (U-$^{13}C_5$, 97-99%)
DLM-487	L-Proline (D_7, 97-98%)
NLM-835	L-Proline (^{15}N, 98%)
CNLM-7822	L-Proline (1-^{13}C, 99%; ^{15}N, 98%)
CNLM-436	L-Proline (U-$^{13}C_5$, 97-99%; ^{15}N, 97-99%)
DNLM-7562	L-Proline (D_7, 98%; ^{15}N, 98%)
CDNLM-6812	L-Proline (U-$^{13}C_5$, 97-99%; U-D_7, 97-99%; ^{15}N, 97-99%)
CLM-1573	L-Serine (1-^{13}C, 99%)
CLM-2013	L-Serine (2-^{13}C, 99%)
CLM-1572	L-Serine (3-^{13}C, 99%)
CLM-1574	L-Serine (U-$^{13}C_3$, 97-99%)
DLM-161	L-Serine (3,3-D_2, 98%)
DLM-582	L-Serine (2,3,3-D_3, 98%)
NLM-2036	L-Serine (^{15}N, 98%)
CNLM-7814	L-Serine (1-^{13}C, 99%; ^{15}N, 98%)
CNLM-474	L-Serine (U-$^{13}C_3$, 97-99%; ^{15}N, 97-99%)
DNLM-6863	L-Serine (2,3,3-D_3, 98%; ^{15}N, 98%)
CDNLM-6813	L-Serine (U-$^{13}C_3$, 97-99%; U-D_3, 97-99%; ^{15}N, 97-99%)
CLM-2261	L-Threonine (U-$^{13}C_4$, 97-99%)
NLM-742	L-Threonine (^{15}N, 98%)
CNLM-7811	L-Threonine (1-^{13}C, 99%; ^{15}N, 98%)
CNLM-587	L-Threonine (U-$^{13}C_4$, 97-99%; ^{15}N, 97-99%)
DNLM-7367	L-Threonine (D_5, 97%; ^{15}N, 98%)
CDNLM-6814	L-Threonine (U-$^{13}C_4$, 97-99%; U-D_5, 97-99%; ^{15}N, 97-99%)
CLM-778	L-Tryptophan (1-^{13}C, 99%)
CLM-1543	L-Tryptophan (indole-2-^{13}C, 98%)
DLM-1092	L-Tryptophan (indole-D_5, 98%)
DLM-6903	L-Tryptophan (D_8, 98%)
NLM-1695	L-Tryptophan (α-^{15}N, 95-99%)
NLM-800	L-Tryptophan ($^{15}N_2$, 98%)
CNLM-7816	L-Tryptophan (1-^{13}C, 99%; α-^{15}N, 98%)
CNLM-2475	L-Tryptophan (U-$^{13}C_{11}$, 97-99%; U-$^{15}N_2$, 97-99%)
DNLM-6904	L-Tryptophan (D_8, 98%; $^{15}N_2$, 98%)
CDNLM-6816	L-Tryptophan (U-$^{13}C_{11}$, 97-99%; U-D_8, 97-99%; U-$^{15}N_2$, 97-99%)

Catalog No.	Description
CLM-776	L-Tyrosine (1-^{13}C, 99%)
CLM-437	L-Tyrosine (2-^{13}C, 99%)
CLM-3378	L-Tyrosine (3-^{13}C, 99%)
CLM-622	L-Tyrosine (phenol-4-^{13}C, 95-99%)
CLM-623	L-Tyrosine (phenol-3,5-$^{13}C_2$, 95-99%)
CLM-1542	L-Tyrosine (ring-$^{13}C_6$, 99%)
CLM-2263	L-Tyrosine (U-$^{13}C_9$, 97-99%)
DLM-2317	L-Tyrosine (3,3-D_2, 98%)
DLM-449	L-Tyrosine (ring-3,5-D_2, 98%)
DLM-451	L-Tyrosine (ring-D_4, 98%)
DLM-589	L-Tyrosine (D_7, 98%)
NLM-590	L-Tyrosine (^{15}N, 98%)
CDLM-2369	L-Tyrosine (ring-$^{13}C_6$, 99%; 3,3-D_2, 30%)
CNLM-439	L-Tyrosine (U-$^{13}C_9$, 97-99%; ^{15}N, 97-99%)
CNLM-7610	L-Tyrosine (2,3-$^{13}C_2$, 99%; ^{15}N, 98%)*
CNLM-7809	L-Tyrosine (1-^{13}C, 99%; ^{15}N, 98%)
DNLM-7373	L-Tyrosine (D_7, 98%; ^{15}N, 98%)
CDNLM-6815	L-Tyrosine (U-$^{13}C_9$, 97-99%; U-D_7, 97-99%; ^{15}N, 97-99%)
CLM-470	L-Valine (1-^{13}C, 99%)
CLM-3050	L-Valine (2-^{13}C, 99%)
CLM-2249	L-Valine (U-$^{13}C_5$, 97-99%)
DLM-7732	L-Valine (3-D, 98%)
DLM-488	L-Valine (D_8, 98%)
NLM-316	L-Valine (^{15}N, 98%)
CNLM-3466	L-Valine (1-^{13}C, 99%; ^{15}N, 98%)
CNLM-8678	L-Valine (2-^{13}C, 99%; ^{15}N, 98%)
CNLM-442	L-Valine (U-$^{13}C_5$, 97-99%; ^{15}N, 97-99%)
DNLM-4643	L-Valine (D_8, 97%; ^{15}N, 97%)
CDNLM-4281	L-Valine (U-$^{13}C_5$, 95-97%; ^{15}N, 96-99%; 2,3-D_2, 97%+)
CDNLM-6817	L-Valine (U-$^{13}C_5$, 97-99%; U-D_8, 97-99%; ^{15}N, 97-99%)

"Over the years, CIL has provided high quality stable isotopically labeled amino acids for our research. Thanks to CIL's leadership in isotope development some unique products, such as 5-fluoro-d_4-deutero-tryptophan or specifically $^{15}N\delta_1$ or $^{15}N\varepsilon_2$ Histidine, have been created. These labels have been instrumental in our efforts to obtain mechanistic details of membrane-embedded ion channels."

Dr. Timothy Cross
National Magnetic Field Laboratory
Florida State University

*For solid-state NMR, see CIL Application Note 22 on page 63.

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Protected Amino Acids

CIL offers a comprehensive list of protected amino acids for use in preparing peptides for use in NMR investigations. The large number of available labeling schemes maximizes the flexibility involved in designing experiments to probe binding, structure, and dynamics of peptides alone or in the presence of small molecules, proteins or nucleic acids. CIL offers amino acids that have N-tert-butoxycarbonyl (*t*-BOC) protecting groups and/or fluorenylmethoxycarbonyl (FMOC) amino-protecting groups.

t-BOC Protected Amino Acids

Catalog No.	Description
CLM-2150	L-Alanine-N-*t*-BOC (1-^{13}C, 99%)
CLM-2011	L-Alanine-N-*t*-BOC (2-^{13}C, 98-99%)
CLM-2151	L-Alanine-N-*t*-BOC (3-^{13}C, 99%)
CLM-3589	L-Alanine-N-*t*-BOC (U-$^{13}C_3$, 97-99%)
DLM-2793	L-Alanine-N-*t*-BOC (3,3,3-D_3, 99%)
NLM-1903	L-Alanine-N-*t*-BOC (^{15}N, 98%)
CNLM-2394	L-Alanine-N-*t*-BOC (U-$^{13}C_3$, 97-99%; ^{15}N, 97-99%)
CNLM-2392	L-Aspartic acid-N-*t*-BOC, β-benzyl ester (U-$^{13}C_4$, 97-99%; ^{15}N, 97-99%)
CLM-1901	L-Cysteine-N-*t*-BOC, S-benzyl (3-^{13}C, 99%)
CLM-2008	L-Glutamic acid-N-*t*-BOC, γ-benzyl ester (1,2-$^{13}C_2$, 99%)
CLM-2152	Glycine-N-*t*-BOC (1-^{13}C, 99%)
CLM-1900	Glycine-N-*t*-BOC (2-^{13}C, 99%)
DLM-2153	Glycine-N-*t*-BOC (2,2-D_2, 98%)
NLM-2109	Glycine-N-*t*-BOC (^{15}N, 98%)
CNLM-2412	Glycine-N-*t*-BOC (U-$^{13}C_2$, 97-99%; ^{15}N, 97-99%)
NLM-2167	L-Isoleucine-N-*t*-BOC (^{15}N, 98%)
CLM-2155	L-Leucine-N-*t*-BOC·H_2O (1-^{13}C, 99%)
CLM-2010	L-Leucine-N-*t*-BOC·H_2O (2-^{13}C, 99%)
DLM-2736	L-Leucine-N-*t*-BOC·H_2O (5,5,5-D_3, 98%)
DLM-3650	L-Leucine-N-*t*-BOC·H_2O (D_{10}, 98%)
NLM-1904	L-Leucine-N-*t*-BOC·H_2O (^{15}N, 98%)
CNLM-2396	L-Leucine-N-*t*-BOC·H_2O (U-$^{13}C_6$, 97-99%; ^{15}N, 97-99%)
CLM-2170	L-Phenylalanine-N-*t*-BOC (1-^{13}C, 99%)
CLM-2061	L-Phenylalanine-N-*t*-BOC (ring-$^{13}C_6$, 99%)
DLM-2157	L-Phenylalanine-N-*t*-BOC (ring-D_5, 98%)
CNLM-2393	L-Phenylalanine-N-*t*-BOC (U-$^{13}C_9$, 97-99%; ^{15}N, 97-99%)
DLM-3651	L-Valine-N-*t*-BOC (D_8, 98%)
NLM-2060	L-Valine-N-*t*-BOC (^{15}N, 98%)

FMOC Protected Amino Acids

CLM-818	L-Alanine-N-FMOC (1-^{13}C, 99%)
CLM-3638	L-Alanine-N-FMOC (2-^{13}C, 99%)
CLM-1142	L-Alanine-N-FMOC (3-^{13}C, 99%)
CLM-7785	L-Alanine-N-FMOC ($^{13}C_3$, 97-99%)
DLM-7316	L-Alanine-N-FMOC (3,3,3-D_3, 98%)
DLM-8168	L-Alanine-N-FMOC (2,3,3,3-D_4, 98%)

Catalog No.	Description
NLM-614	L-Alanine-N-FMOC (^{15}N, 98%)
CNLM-4355	L-Alanine-N-FMOC (U-$^{13}C_3$, 97-99%; ^{15}N, 97-99%)
CDNLM-7852	L-Alanine-N-FMOC (U-$^{13}C_3$, 97-99%; U-D_4, 97-99%; ^{15}N, 97-99%)
CLM-8475-H	L-Arginine-N-FMOC, Pbf (U-$^{13}C_6$, 99%) (CP 90-95%) (contains solvent)
NLM-8841	L-Arginine-N-FMOC, Pbf (U-$^{15}N_4$, 98%) (contains solvent)
CNLM-8474-H	L-Arginine-N-FMOC, Pbf (U-$^{13}C_6$, 99%; U-$^{15}N_4$, 99%) (contains solvent)
NLM-4204	L-Asparagine-N-FMOC, N-β-trityl ($^{15}N_2$, 98%)
CNLM-4354	L-Asparagine-N-FMOC (U-$^{13}C_4$, 97-99%; U-$^{15}N_2$, 97-99%)
CNLM-6193	L-Asparagine-N-FMOC, N-β-trityl (U-$^{13}C_4$, 97-99%; U-$^{15}N_2$, 97-99%)
CNLM-4788	L-Aspartic acid-N-FMOC (U-$^{13}C_4$, 97-99%; ^{15}N, 97-99%)
CNLM-4752	L-Aspartic acid-N-FMOC, β-*O*-tert-butyl ester ($^{13}C_4$, 97-99%; ^{15}N, 97-99%)
DLM-4721	L-Cysteine-N-FMOC, S-trityl (3,3-D_2, 98%)
CNLM-4722	L-Cysteine-N-FMOC, S-trityl (U-$^{13}C_3$, 97-99%; ^{15}N, 97-99%)
CNLM-4356	L-Glutamine-N-FMOC (U-$^{13}C_5$, 97-99%; U-$^{15}N_2$, 97-99%)
CNLM-7252	L-Glutamine-N-FMOC, N-γ-trityl (U-$^{13}C_5$, 97-99%; U-$^{15}N_2$, 97-99%)
CNLM-4753	L-Glutamic acid-N-FMOC, γ-tert-butyl ester (U-$^{13}C_5$, 97-99%; ^{15}N, 97-99%) (CP 96%)
CLM-3639	Glycine-N-FMOC (1-^{13}C, 99%)
CLM-2387	Glycine-N-FMOC (2-^{13}C, 99%)
CLM-7547	Glycine-N-FMOC ($^{13}C_2$, 97-99%)
DLM-7339	Glycine-N-FMOC (2,2-D_2, 98%)
NLM-694	Glycine-N-FMOC (^{15}N, 98%)
CNLM-7697	Glycine-N-FMOC (1-^{13}C, 99%; ^{15}N, 98%)
CNLM-4357	Glycine-N-FMOC (U-$^{13}C_2$, 97-99%; ^{15}N, 97-99%)
CDNLM-7853	Glycine-N-FMOC (U-$^{13}C_2$, 97-99%; 2,2-D_2, 97-99%; ^{15}N, 97-99%)
NLM-8010	L-Histidine-N-FMOC, N-Im-trityl ($^{15}N_3$, 98%)
CLM-8043	L-Isoleucine-N-FMOC (1-^{13}C, 99%)
CLM-7794	L-Isoleucine-N-FMOC (U-$^{13}C_6$, 97-99%)
NLM-391	L-Isoleucine-N-FMOC (^{15}N, 98%)
CNLM-4346	L-Isoleucine-N-FMOC (U-$^{13}C_6$, 97-99%; ^{15}N, 97-99%)

Protected Amino Acids

Catalog No.	Description
CLM-3691	L-Leucine-N-FMOC (1-^{13}C, 99%)
CLM-7546	L-Leucine-N-FMOC (1,2-$^{13}C_2$, 99%)
CLM-3683	L-Leucine-N-FMOC (U-$^{13}C_6$, 97-99%)
DLM-7202	L-Leucine-N-FMOC (5,5,5-D_3, 98%)
DLM-7575	L-Leucine-N-FMOC (D_{10}, 98%)
NLM-2397	L-Leucine-N-FMOC (^{15}N, 98%)
CNLM-4345	L-Leucine-N-FMOC (U-$^{13}C_6$, 97-99%; ^{15}N, 97-99%)
CLM-6194	L-Lysine-α-N-FMOC, ε-N-*t*-BOC (1-^{13}C, 99%)
CLM-7865-H	L-Lysine-α-N-FMOC, ε-N-*t*-BOC (U-$^{13}C_6$, 99%)
NLM-4631	L-Lysine-α-N-FMOC, ε-N-*t*-BOC ($^{15}N_2$, 96-98%)
CNLM-4754-H	L-Lysine-α-N-FMOC, ε-N-*t*-BOC (U-$^{13}C_6$, 99%; U-^{15}N2, 99%)
CLM-8166	L-Methionine-N-FMOC (1-^{13}C, 99%)
CLM-1141	L-Methionine-N-FMOC (methyl-^{13}C, 99%)
NLM-4632	L-Methionine-N-FMOC (^{15}N, 98%) (CP 95%)
CNLM-4358	L-Methionine-N-FMOC (U-$^{13}C_5$, 97-99%; ^{15}N, 97-99%)
CLM-4824	L-Phenylalanine-N-FMOC (1-^{13}C, 99%)
CLM-3684	L-Phenylalanine-N-FMOC (ring-$^{13}C_6$, 99%)
DLM-7786	L-Phenylalanine-N-FMOC (ring-D_5, 98%)
NLM-1265	L-Phenylalanine-N-FMOC (^{15}N, 98%)
CNLM-4362	L-Phenylalanine-N-FMOC (U-$^{13}C_9$, 97-99%; ^{15}N, 97-99%)

Catalog No.	Description
CLM-8044	L-Proline-N-FMOC (1-^{13}C, 99%)
NLM-3906	L-Proline-N-FMOC (^{15}N, 98%)
CNLM-4347	L-Proline-N-FMOC (U-$^{13}C_5$, 97-99%; ^{15}N, 97-99%)
CLM-8167	L-Serine-N-FMOC, *O*-*t*-butyl ether (1-^{13}C, 99%)
NLM-4630	L-Serine-N-FMOC, *O*-*t*-butyl ether (^{15}N, 98%)
CNLM-8403	L-Serine-N-FMOC (U-$^{13}C_3$, 97-99%; ^{15}N, 97-99%)
CNLM-4755	L-Serine-N-FMOC, *O*-*t*-butyl ether (U-$^{13}C_3$, 97-99%; ^{15}N, 97-99%) (3% D Isomer)
NLM-8170	L-Threonine-N-FMOC, *O*-*t*-butyl ether (^{15}N, 98%)
CNLM-7615	L-Threonine-N-FMOC, *O*-*t*-butyl ether (U-$^{13}C_4$, 97-99%; ^{15}N, 97-99%)
DLM-6113	L-Tryptophan-N-FMOC (indole-D_5, 98%)
CNLM-6077	L-Tryptophan-N-FMOC (U-$^{13}C_{11}$, 97-99%; U-$^{15}N_2$, 97-99%)
NLM-8169	L-Tyrosine-N-FMOC, *O*-*t*-butyl ether (^{15}N, 98%)
CNLM-4349	L-Tyrosine-N-FMOC, *O*-*t*-butyl ether (U-$^{13}C_9$, 97-99%; ^{15}N, 97-99%)
CLM-3640	L-Valine-N-FMOC (1-^{13}C, 99%)
CLM-7793	L-Valine-N-FMOC (U-$^{13}C_5$, 97-99%)
DLM-7784	L-Valine-N-FMOC (D_8, 98%)
NLM-4243	L-Valine-N-FMOC (^{15}N, 98%)
CNLM-4348	L-Valine-N-FMOC (U-$^{13}C_5$, 97-99%; ^{15}N, 97-99%)

"Over the past two decades, companies providing specifically labeled isotopes and precursors have changed our ways and understanding of biomolecular NMR sample preparations. CIL, being the widely popular company, certainly plays a crucial role in this development. I enjoy working with CIL because of the value, quality of products and superb service. I have been ordering from them for the past ten years: from Munich as a Ph.D. student, from San Francisco as post-doc, and from Hyderabad as a PI. To tell you the truth, I do not want to even imagine an NMR world without isotopes and CIL."

Dr. Mandar V. Deshmukh
Scientist, Structural Biology (NMR)
Centre for Cellular and Molecular Biology

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Sparse Labeling Media and Reagents

The presence of one bond ^{13}C-^{13}C coupling in proteins may present difficulties in obtaining structural information in both solid-state and solution NMR. It is easier to overcome these difficulties by simply analyzing sparsely ^{13}C-labeled protein than resorting to using specialized pulse sequences. Sparsely labeled protein is made by expressing protein in *E. coli* grown using selectively ^{13}C-labeled carbon sources. The use of 2-^{13}C and 1,3-$^{13}C_2$ glycerol, as originally described by LeMaster[1], remains to be the most popular sparse labeling method in current use. Proteins expressed using 1-^{13}C glucose and 2-^{13}C glucose as sole carbon sources, however, have recently been used to determine intermolecular distances in supramolecular complexes using ^{13}C-^{13}C solid state NMR.[2]

CIL offers the following selectively ^{13}C-labeled *E. coli* carbon sources and rich media. The suffix "-S" in catalog numbers for BioExpress® 1000 media refers to a 10 mL packaged size that is used to prepare 100 mL of formulated media.

Sparse Labeling Reagents and Media

Catalog No.	Description
CGM-1000-CN-25-S	BioExpress® 1000 (U-^{13}C, 25%; U-^{15}N, 98%) (10x Concentrate)
CGM-1000-CN-25	BioExpress® 1000 (U-^{13}C, 25%; U-^{15}N, 98%) (10x Concentrate)
CGM-1000-CN-35-S	BioExpress® 1000 (U-^{13}C, 35%; U-^{15}N, 98%) (10x Concentrate)
CGM-1000-CN-35	BioExpress® 1000 (U-^{13}C, 35%; U-^{15}N, 98%) (10x Concentrate)
CGM-1000-CN-45-S	BioExpress® 1000 (U-^{13}C, 45%; U-^{15}N, 98%) (10x Concentrate)
CGM-1000-CN-45	BioExpress® 1000 (U-^{13}C, 45%; U-^{15}N, 98%) (10x Concentrate)
CLM-420	D-Glucose (1-^{13}C, 99%)
CLM-746	D-Glucose (2-^{13}C, 98-99%)
CLM-1396-25-1	D-Glucose (U-$^{13}C_6$, 24-25%)
CLM-1397	Glycerol (2-^{13}C, 99%)
CLM-1857	Glycerol (1,3-$^{13}C_2$, 99%)

References

1. LeMaster, D.M. **1996**. Protein Structure Refinement Using $^{13}C\alpha$ Chemical Shift Tensors. *J Am Chem Soc, 118*, 9255-9264.
2. Loquet, A.; Giller, K.; Becker, S.; and Lange, A. **2010**. Supramolecular interactions probed by ^{13}C-^{13}C solid-state NMR spectroscopy. *J Am Chem Soc, 132*, 15164-15166.

Isotope Labeling of Proteins for NMR Spectrometry

Stanley J. Opella, Ph.D.
Department of Chemistry, University of California, San Diego, La Jolla, California

The design and execution of NMR experiments on proteins starts with isotopic labeling. The goal is to use only one or a few uniformly labeled samples for all of the necessary NMR experiments, even though specific or selective (by residue) type labeling may be necessary at the early stages of an investigation and to solve particularly vexing problems along the way to structure determination. In general, the first consideration in both solution NMR and solid-state NMR is the broadening effects of homonuclear dipole-dipole couplings. In some cases, this can be accomplished with complex pulse sequences, but a more general solution is to employ various strategies for the dilution of the nuclei of interest through isotopic labeling. This was demonstrated at the very beginning of solution NMR of proteins through the use of extensive deuteration,[1] leaving only a few ^{1}H nuclei behind for detection as isolated nuclei at known sites. The initial high-resolution magic angle spinning (MAS) solid-state NMR of proteins was performed on samples with natural abundance of ^{13}C (1%), which effectively isolated the ^{13}C nuclei from each other.[2] Of course, this was at the cost of sensitivity for the experiments. Among the first assignment strategies for ^{13}C NMR of proteins in solution involved the use of 35% uniformly ^{13}C-labeled proteins, where a balance was struck between increasing the sensitivity and line broadening.[3]

The original implementation of 100% uniform ^{15}N labeling of proteins for solid-state NMR experiments exploited the advantage of its natural chemical dilution, since there are no nitrogens directly bonded to another nitrogen in a protein, and three bonds and two carbon atoms separate the crucial amide

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Sparse Labeling Media and Reagents

Isotope Labeling of Proteins for NMR Spectrometry

backbone nitrogens from each other. As a result 100% uniformly ^{15}N-labeled proteins has been widely used following the original demonstrations in solution NMR,[4] oriented sample (OS) solid-state NMR,[5] and MAS solid-state NMR[6] without the need for additional spectroscopic manipulations.

Nonetheless, there are advantages to performing triple-resonance experiments on 100% uniformly ^{13}C- and 100% uniformly ^{15}N-labeled proteins, and this has led to the modern era of high-throughput and high-precision structure determination. By having all backbone atoms labeled, assignment schemes based on "walking" up the backbone are feasible, and have been widely used since their original introductions in solution NMR[7] and MAS solid-state NMR of proteins.[8] As long as the motions intrinsic to these experiments average the ^{13}C-^{13}C homonuclear dipole-dipole couplings, they can be used in a controlled way for assignment purposes and for ^{13}C detection, which has substantially greater sensitivity than ^{15}N detection. In some cases, partial dilution of the ^{13}C distribution in the protein through using specifically labeled glycerols or glucose yields improvements in resolution.[9,10]

The most serious problem occurs with OS solid-state NMR where there are no motions to average the dense network of ^{13}C-^{13}C homonuclear couplings among the backbone and side chain sites. The couplings interfere with routine solid-state NMR experiments, and make ^{13}C detection difficult to implement, with essentially the only recourse being to develop multiple-pulse sequences to decouple the ^{13}C-^{13}C homonuclear couplings; however, this is generally quite costly in terms of sensitivity because of the need to sample the signals within the short windows of the pulse sequence and the attendant filtering problems. An alternative is to tailor the isotopic labeling so that the protein is 100% uniformly labeled with ^{15}N and 35% randomly uniformly labeled with ^{13}C.[11,12] This provides sufficient spatial isolation to minimize broadening due to ^{13}C-^{13}C couplings, while still providing a large gain in sensitivity over natural abundance ^{13}C detection and over ^{15}N detection due to its higher gyromagnetic ratio.

The field of NMR of proteins has advanced considerably since the first spectra were reported in solution[13] and the solid state.[2] This can be attributed in approximately equal proportions to advances in instrumentation, advances in experimental methods, and to the advances in isotopic labeling that are briefly outlined here. Importantly, the reagents for these isotopic labeling approaches are now commercially available, greatly increasing the feasibility of their implementation in laboratories throughout the world.

References

1. Markley, J.L.; Putter, I.; Jardetzky, O. **1968**. High-resolution nuclear magnetic resonance spectra of selectively deuterated staphylococcal nuclease. *Science, 161,* 1249-1251.
2. Opella, S.J.; Frey, M.H.; Cross, T.A. **1979**. Detection of individual carbon resonances in solid proteins. *J Am Chem Soc, 101*, 5856-5857.
3. Stockman, B.J.; Westler, W.M.; Darba, P., Markley, J.L. **1988**. Detailed analysis of carbon-13 NMR spin systems in a uniformly carbon-13 enriched protein: Flavodoxin from Anabaena 7120. *J Am Chem Soc, 110*, 4095-4096.
4. Bogusky, M.J.; Leighton, P.; Schiksnis, R.A.; Khoury A.; Lu, P.; Opella, S.J. **1990**. ^{15}N NMR spectroscopy of proteins in solution. *J Magn Reson, 86*, 11-29.
5. Cross, T.A.; Tsang, P.; Opella, S.J. **1983**. Comparison of protein and deoxyribonucleic acid backbone structures in fd and Pf1 bacteriophages. *Biochemistry, 22*, 721-6.
6. Cross, T.A.; DiVerdi, J.A.; Opella, S.J. **1982**. Strategy for nitrogen NMR in biopolymers. *J Am Chem Soc, 104*, 1759-1761.
7. Ikura, M.; Kay, L.E.; Bax, A. **1990**. A novel approach for sequential assignment of ^{1}H, ^{13}C, and ^{15}N spectra of larger proteins: Heteronuclear triple-resonance three-dimensional NMR spectroscopy. Application to Calmodulin. **1990**. *Biochemistry, 29*, 4659-4667.
8. Rienstra, C.M.; Tucker-Kellog, L.; Jaroniec, C.P.; Hohwy, M.; Reif, B.; McMahon, M.T.; Tidor, B.; Lozano-Perez, T.; Griffin, R.G. **2002**. *De novo* determination of peptide structure with solid-state magic-angle spinning NMR spectroscopy. *Proc Natl Aca Sci USA, 99*, 10260-5.
9. Hong, M. **1999**. Determination of multiple phi-torsion sangles in proteins by selective and extensive ^{13}C labeling and two-dimensional solid-state NMR. *J Magn Reson, 139*, 389-401.
10. Castellani, F.; van Rossum, B.; Diehl, A.; Schubert, M.; Rhbein, K.; Oschkinat, H. **2002**. Structure of a protein determined by solid-state magic-angle-spinning NMR spectroscopy. *Nature, 420*, 98-102.
11. Sinha, N.; Filipp, F.V.; Jairam, L.; Park, S.H.; Bradley, J.; Opella, S.J. **2007**. Tailoring ^{13}C labeling for triple-resonance solid-state NMR experiments on aligned samples of proteins. J *Magn Reson Chem, 45*, 5107-5115.
12. Filipp, F.V.; Sinha, N.; Jairam, L.; Bradley, J.; Opella, S.J. **2009**. Labeling strategies for ^{13}C-detected aligned-sample solid-state NMR of proteins. *J Magn Reson, 201*, 121-130.
13. Saunders, M.; Wishnia, A.; Kirkwood, J.G. **1957**. The nuclear magnetic resonance spectrum of ribonuclease. *J Am Chem Soc, 79*, 3289 - 3290.

Deuterated Detergents and Phospholipids for Membrane Proteins

Membrane proteins can be divided into three categories:

1. Integral membrane proteins, which penetrate the lipid bilayer;
2. Peripheral membrane proteins, which are external and bound through non-covalent interactions; and
3. Lipid-anchored proteins, which are external, but bound with covalent bonds.

There is great interest in determining structure of integral membrane proteins due to the importance of these proteins in participating in cellular processes. Despite the significant functional importance of membrane proteins, the structural biology has been particularly challenging as shown by the low number of membrane protein structures determined.[1]

The determination of structure and dynamics of membrane proteins using NMR requires samples containing protein that is properly folded. Fortunately, membrane proteins often keep native-like structures in detergent micelles. Deuterated solubilization agents such as detergents often make NMR investigations easier compared to using unlabeled agents. In some cases, as in methyl labeling, deuterated reagents of this type are required. CIL is pleased to offer the following deuterated detergents and phospholipid agents for use with membrane proteins.

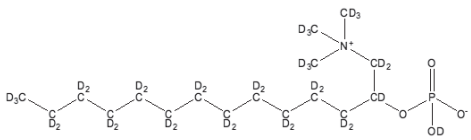
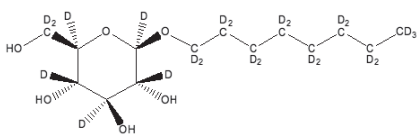
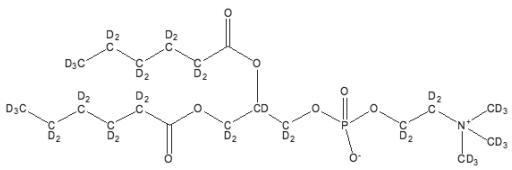
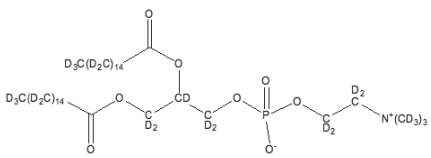
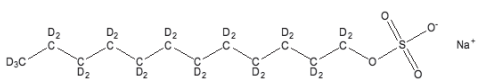
Reference

1 There are 263 unique membrane protein structures as of Feb 6, 2011. See http://blanco.biomol.uci.edu/index.shtml for more information.

"CIL has been a strong supporter of NMR methods of development over the years, providing critical isotope-enriched reagents for research and development, without which many of the recent advances in biomolecular NMR would simply not have been possible. In particular, the broad biological impact and tremendous success of the multidimensional triple-resonance biomolecular NMR would not have been achieved without the high quality and broadly accessible reagents that CIL has provided to the scientific community over the last 20 years."

Dr. Gaetano Montelione
Professor of Molecular Biology and Biochemistry
Rutgers University Director of the
Northeast Structural Genomics Consortium

Deuterated Detergents and Phospholipids

Catalog No.	Description
DLM-2274	Dodecylphosphocholine (D_{38}, 98%) 
DLM-6726	*N*-Octyl β-Glucoside (D_{24}, 98%) 
DLM-4341	DL-A-Phosphatidylcholine, dihexanoyl (D_{40}, 98%) (DHPC) (CP 95%) 
DLM-8256	DL-A-Phosphatidylcholine, dipalmitoyl (DPPC) (U-D_{80}, 98%) (CP 95%+) 
DLM-197	Sodium dodecyl sulfate (D_{25}, 98%) 

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Deuterated Detergents and Phospholipids for Membrane Proteins


The Role of Deuterated Detergents in NMR Structure Determination of Membrane Proteins

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Membrane protein structural biology relies heavily on detergents for solubility and stability. To date, all polytopic membrane protein structures determined with NMR were prepared in detergents. In addition to water, the detergents contribute to solvent signals. Yet, unlike water, detergentshave several aliphatic proton resonances between 0 and 3 ppm that can interfere with protein aliphatic proton detection. Suppression of these signals is difficult and not commonly performed in the pulsed sequences used.

For NMR structure determination of large systems such as protein-detergent complexes, proteins are perdeuterated to reduce relaxation rates and, thereby, increase sensitivity and resolution for triple-resonance experiments. However, important NOE-derived distance restraints are lost as a result of the deuteration, dramatically affecting the resolution of the structures calculated. To acquire additional NOE distance restraints, specific amino acid labeling and methyl labeling is often employed. The aliphatic proton peaks of these amino acids overlap with the detergent signals, which are orders of magnitude more abundant than the protein, and significantly interfere with the NMR experiment.[1] In these cases, deuterated detergents have proven to be vital to the detection of the protein aliphatic protons.[2-4] Ligands and cofactors also have protons that overlap with detergent signals and, in order to determine the structure of the bound ligand, deuterated detergents have been utilized.[4]

References

1. Sanders, C.R.; and Sonnichsen, F. **2006**. Solution NMR of membrane proteins: practice and challenges. *Magn Reson Chem, 44* Spec No, S24-40.
2. Hiller, S.; Garces, R.G.; Malia, T.J.; Orekhov, V.Y.; Colombini, M.; and Wagner, G. **2008**. Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. *Science, 321,* 1206-1210.
3. Hwang, P.M.; Choy, W.Y.; Lo, E.I.; Chen, L.; Forman-Kay, J.D.; Raetz, C.R.; Prive, G.G.; Bishop, R.E.; and Kay, L.E. **2002**. Solution structure and dynamics of the outer membrane enzyme PagP by NMR. *Proc Natl Acad Sci USA, 99*, 13560-13565.
4. Zhou, Y.; Cierpicki, T.; Jimenez, R.H.; Lukasik, S.M.; Ellena, J.F.; Cafiso, D.S.; Kadokura, H.; Beckwith, J.; and Bushweller, J.H. **2008**. NMR solution structure of the integral membrane enzyme DsbB: functional insights into DsbB-catalyzed disulfide bond formation. *Mol Cell, 31*, 896-908.

"The methodological development that NMR has seen in the past six decades would not have been possible without close collaborations with companies that love to see progress happening. CIL is a particularly strong and supportive partner, and many ideas with respect to labeling patterns that looked crazy on the first sight became reality through CIL, enabling us to conquer new areas for NMR structure determination. There is a wide variety of examples where talking to CIL has pushed science forward."

Dr. Hartmut Oschkinat
Department of NMR – Supported Structural Biology
FMP-Berlin

Miscellaneous Protein Reagents

Reverse Micelles

NMR spectroscopy of encapsulated proteins dissolved in low viscosity fluids is emerging as a powerful supplement to traditional solution NMR approaches. Originally developed to overcome the slow tumbling problem presented by large soluble proteins,[1] the general approach of using reverse micelles has now seen applications in the study of integral[2] and anchored[3] membrane proteins; proteins of marginal stability;[4] protein structure,[5] dynamics[6] and hydration.[7] Nucleic acids have also been successfully investigated in this manner.[8] The distinguishing feature of this approach is the nature of the sample. Spontaneously formed reverse micelles are the dominant vehicle for encapsulation and the low-viscosity short-chain alkane fluids are the dominant solvent with liquid propane and ethane being the most desirable.[9] The availability of deuterated surfactants, co-surfactants and alkane solvents avoids the complications of large unwanted ^{1}H resonances that would greatly interfere with multidimensional NMR of encapsulated biopolymers in low-viscosity solvents.

References

1 Wand, A.J.; Ehrhardt, M.R.; and Flynn, P.F. **1998**. High-resolution NMR of encapsulated proteins dissolved in low-viscosity fluids. *Proc Natl Acad Sci USA, 95*, 15299-15302.
2 Kielec, J.M.; Valentine, K.G.; Babu, C.R.; and Wand, A.J. **2009**. Reverse micelles in integral membrane protein structural biology by solution NMR spectroscopy. *Structure, 17*, 345-351.
3 Valentine, K.G.; Peterson, R.W.; Saad, J.S.; Summers, M.F.; Xu, X.; Ames, J.B.; and Wand, A.J. **2010**. Reverse micelle encapsulation of membrane-anchored proteins for solution NMR studies. *Structure, 18*, 9-16.
4 Peterson, R.W.; Anbalagan, K.; Tommos, C.; and Wand, A.J. **2004**. Forced folding and structural analysis of metastable proteins. *J Am Chem Soc, 126*, 9498-9.
5 Babu, C.R.; Flynn, P.F.; and Wand, A.J. **2001**. Validation of protein structure from preparations of encapsulated proteins dissolved in low-viscosity fluids. *J Am Chem Soc, 123*, 2691-2692.
6 Simorellis, A.K. and Flynn, P.F. **2006**. Fast local backbone dynamics of encapsulated ubiquitin. *J Am Chem Soc, 128*, 9580-9581.
7 Nucci, N.V.; Pometun, M.S.; and Wand, A.J. **2011**. Site-resolved measurement of water-protein interactions by solution NMR. *Nat Struct Mol Biol, 245-249*.
8 Workman, H. and Flynn, P.F. **2009**. Stabilization of RNA Oligomers through Reverse Micelle Encapsulation. *J Am Chem Soc, 131*, 3806-3807.
9 Peterson, R.W.; Lefebvre, B.G.; and Wand, A.J. **2005**. High-resolution NMR studies of encapsulated proteins in liquid ethane. *J Am Chem Soc, 127*, 10176-10177.

Reagents for Reverse Micelles

Catalog No.	Description
DLM-276	Ethane (D_6, 98%)
DLM-8117	Hexadecyltrimethylammonium bromide (D_{42}, 98%)
DLM-8840	Hexadecyltrimethylammonium chloride (D_{42}, 98%)
DLM-691	*n*-Hexanol (D_{13}, 98%)
DLM-1213	*n*-Pentane-D_{12} (D, 98%)
DLM-3476	Propane (1,1,1,3,3,3-D_6, 98%)

Deuterated Reagents

Catalog No.	Description
DLM-710	Ammonium deuteroxide (D_5, 99%) (~25% ND_4OD in D_2O)
DLM-54	Deuterium chloride (D, 99.96%) (~20% DCL in D_2O)
DLM-414	EDTA (D_{12}, 98%)
DLM-3908	EDTA (D_{16}, 98%)
DLM-2622	DL-1,4-Dithiothreitol (DTT) (D_{10}, 98%)
DLM-6686	Tris(2-carboxyethyl)phosphine•DCl (TCEP) (D_{16}, 98%)
DLM-2713	2-Mercaptoethanol (D_6, 98%)

Dynamic Nuclear Polarization Reagent

^{13}C-depleted, uniformly deuterated glycerol is used to reduce ^{13}C background signals in MAS spectra of cryoprotected proteins, particularly in solid-state DNP applications pioneered by the laboratory of Dr. Robert Griffin (Francis Bitter Magnet Laboratory, MIT) and commercialized by Bruker BioSpin.

Catalog No.	Description
CDLM-8660	Glycerol ($^{12}C_3$, 99.9+%; D_8, 98%)

^{17}O NMR Reagents

Recently, the laboratory of Dr. Gang Wu (Queens University, Ontario, Canada) reported the use of Quadrupole Central Transition (QCT) ^{17}O NMR spectroscopy to probe large protein-ligand complexes in solution.[1] They demonstrated that ^{17}O-labeled ligands at ~30% ^{17}O enrichment can be detected in protein-ligand complexes of 240 kDa with <1 mM protein concentrations, but expect the technique to work with complexes as large as 400-500 kDa when using ^{17}O enrichments as high as 90%. They also report QCT ^{17}O NMR spectroscopy should be applicable to a wide variety of biological macromolecules. Additionally, this laboratory reports the first comprehensive solid-state ^{17}O MAS NMR of large protein-ligand complexes.[2] CIL is proud to offer highly enriched ^{17}O water as a starting material to probe protein-ligand complexes using either QCT ^{17}O NMR or solid-state ^{17}O MAS NMR.

Catalog No.	Description
OLM-782-70	Water (^{17}O, 70%)
OLM-782-85	Water (^{17}O, 85%)
OLM-782-90	Water (^{17}O, 90%)

References

1 Zhu, J. and Wu, G. **2011**. Quadrupole central transition ^{17}O NMR spectroscopy of biological macromolecules in aqueous solution. *J Am Chem Soc, 133*, 920-932.
2 Zhu, J.; Ye, E.; Terskikh, V.; and Wu, G. **2010**. Solid-state ^{17}O NMR spectroscopy of large protein-ligand complexes. *Angew Chem Int Ed, 49*, 8399-8402.

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Miscellaneous Protein Reagents

Protein Stabilization Reagent

Dr. Kaori Wakamatsu from Gunma University (Gunma, Japan) has pioneered the use of choline-*O*-sulfate (COS) and uniformly deuterated choline-*O*-sulfate (COS-D_{13}) for use in protein NMR studies. His results show significant improvement in stabilizing soluble forms of protein in solution with a concurrent increase in sensitivity in multidimensional NMR data sets acquired at near-human physiological temperatures.

Advantages of COS:

- Prevention of thermal denaturation of membrane proteins, including GPCRs
- Prevention of precipitation of protein and protein/peptide complexes
- Facilitation of NMR measurements, especially at elevated temperatures
- Improvement of protein recovery during purification

CIL is proud to offer unlabeled COS for the world-wide protein NMR community at a reasonable price. Please ask your local sales representative for a quote for uniformly deuterated COS (COS-D_{13}).

Catalog No.	Description
ULM-8703	Choline-*O*-sulfate (unlabeled)

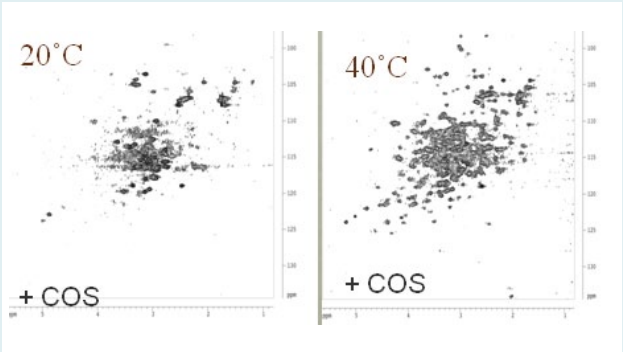

Figure 2. HSQC spectra of uniformly ^{15}N labeled Gi1α in the presence of 1 M COS at 20°C (left) and 40°C (right). Spectra were recorded on a Bruker ARX-400 spectrometer.

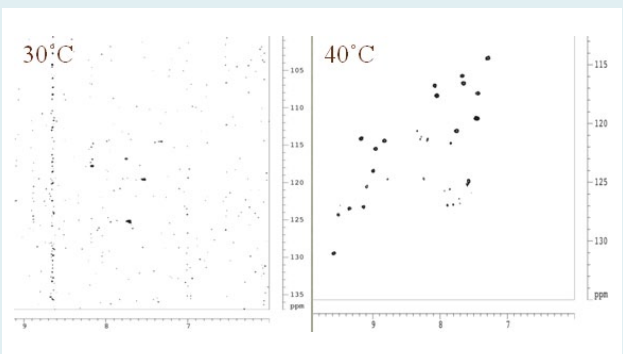

Figure 3. HSQC spectra of Gi1α labeled with [^{15}N] phenylalanine in the presence of 1 *M* COS-d_{13} at 30°C (left) and 40°C (right). Almost all (18 out of 19) phenylalanine signals are clearly observed at 40°C. Spectra were recorded on a Bruker Avance-700 spectrometer.

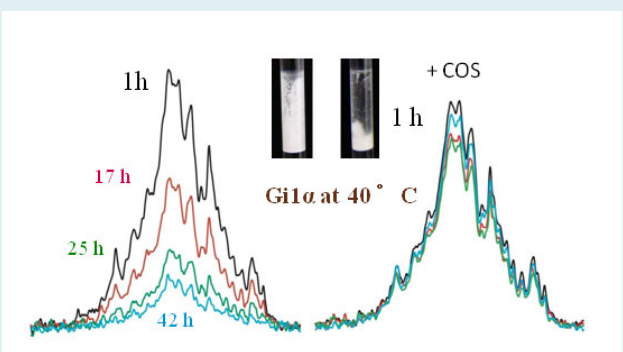

Figure 1. 1D HSQC spectra of uniformly ^{15}N-labeled Gi1α in the absence (left) or presence of 1 *M* COS (right) were recorded on a Bruker ARX-400 spectrometer at 40°C at 1 h (black), 17 h (red), 25 h (green), and 42 h (cyan) after temperature control. The photographs demonstrate much less precipitate in the presence of COS at 42 h.

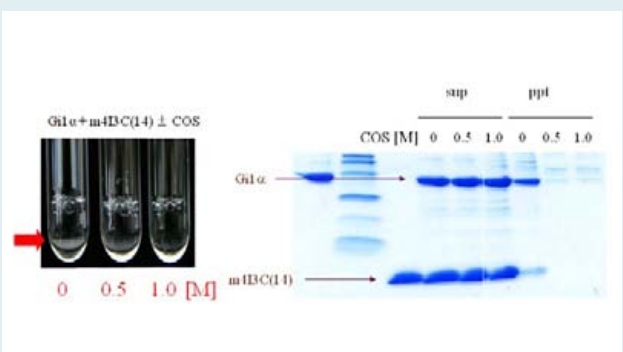

Figure 4. COS prevents co-precipitation of Gi1α and its selective activator, m4I3C(14), on mixing. Gi1α and m4I3C(14) form precipitates on mixing (photograph at bottom, left tube). The composition of the precipitates is confirmed by SDS-PAGE (bottom right panel, lane ppt/0). In the presence of COS, the precipitates are not observed (photograph, SDS-PAGE).

Protein NMR Standards

CIL offers several labeled protein standards that are used to test new pulse sequences and monitor spectrometer performance. Protein standards are available for both solution and solid-state NMR. All CIL protein standards exhibit excellent long-term stability when stored properly. These products are offered as either concentrated solutions ("-S" in the catalog number) or as a bulk solid sealed in Eppendorf tubes or small vials. Please indicate if you would like the solution (550 µL) prepackaged in a 5 mm NMR tube.

- The Chicken α-Spectrin SH3 Domain is available in microcrystalline form (in an ammonium sulfate emulsion) or as a 9 mg/mL solution (10% D2O/90% H_2O containing 0.02% NaN_3, pH 3.5).
- Ubiquitin is offered as the free protein and in a His-tagged form. It is offered as a powder.
- GB1 is offered as a N-terminal TEV-cleavable 6xHis-tag as a 1.5 mM solution (X PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.76 mM KH_2PO4), 0.02% NaN_3, 0.1 mM TSP, in 10% D_2O/90% H_2O, pH 6.5). GB1 is noted for its excellent thermal stability.

A full technical data package containing 2D NMR data and peak assignments accompanies every order for the Chicken α-Spectrin SH3 Domain and GB1.

See your local sales representative if you would like to obtain either unlabeled or ^{15}N-labeled GB1 with the 6xHis tag for use in optimizing conditions for cleaving the 6x His-tag. Also please ask your sales representative about the availability of microcrystalline GB1 for solid-state NMR.

Catalog No.	Description
CLM-8227	SH3 Domain Protein (U-^{13}C, 98%)
NLM-6839	SH3 Domain Protein (U-^{15}N, 98%)
NLM-6839-S	SH3 Domain Protein (U-^{15}N, 98%) (9 mg/mL solution)
CNLM-6840	SH3 Domain Protein (U-^{13}C, 98%; U-^{15}N, 98%) (Microcrystalline Slurry)
CNLM-6840-S	SH3 Domain Protein (W/ 0.01% sodium azide) (U-^{13}C, 98%; U-^{15}N, 98%) (9 mg/mL solution)
CDNLM-6841	SH3 Domain Protein (U-^{13}C, 98%; U-D, 98%; U-^{15}N, 98%)
CDNLM-6841-S	SH3 Domain Protein (9 mg/mL solution) (U-^{13}C, 98%; U-D, 98%; U-^{15}N, 98%)
NLM-4318	Ubiquitin (U-^{15}N, 98%+)
CNLM-4215	Ubiquitin (U-^{13}C, 99%; U-^{15}N, 95-99%)
CNLM-4215-1	Custom Ubiquitin Standard (U-^{13}C, 99%; U-^{15}N, 95-99%)
CDNLM-4319-70	Ubiquitin (U-^{13}C, 99%; U-D, 70%; U-^{15}N, 95-99%)
CDNLM-4319-98	Ubiquitin (U-^{13}C, 99%; U-D, 98%; U-^{15}N, 95-99%)
ULM-4316	Ubiquitin (Unlabeled)
NLM-7361	Ubiquitin (Algal) (U-^{15}N, 98%)
CNLM-7362	Ubiquitin (Algal) (U-^{13}C, 98%; U-^{15}N, 98%)
CNLM-2408	GFL Peptide Standard (^{13}C, 98%; ^{15}N, 96-99%) 1 mM in DMSO-d_6

His-Tagged Standards

CLM-8418	His-Ubiquitin (Human) (^{13}C, 98%+)
DLM-8417	His-Ubiquitin (Human) (D, 97%+)
NLM-8419	His-Ubiquitin (Human) (^{15}N, 98%+)
CDLM-7582-SL-1	His-Ubiquitin DC (^{13}C, 98%; D, 98%)
CNLM-8420	His-Ubiquitin (Human) (^{13}C, 98%+; ^{15}N, 98%+)
DNLM-7583	His-Ubiquitin (Human) (U-D, 97%; U-^{15}N, 98%)
DNLM-7583-SL-1	His Ubiquitin DN (D, 98%; ^{15}N, 98%)
CDNLM-8421	His-Ubiquitin (Human) (^{13}C, 98%+; D, 97%+; ^{15}N, 98%+)
ULM-8416	His-Ubiquitin (Human) (Unlabeled)
New! CNLM-8663	His-GB1 (^{13}C, 98%+; ^{15}N, 98%+) (1.5 mM in PBS, pH 6.5, 0.02% sodium azide)

Product	Amino Acid Length	Amino Acid Sequence
Algal Ubiquitin	76 residues	MQIFVKTLTG KTITLEVESS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLADYN IQKESTLHLV LRLRGG
Human Ubiquitin	76 residues	MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRGG
Chicken α-Spectrin SH3 Domain	62 residues	MDETGKELVL ALYDYQEKSP REVTMKKGDI LTLLNSTNKD WWKVEVNDRQ GFVPAAYVKK LD
His6x-GB1 (β-1 immunoglobulin domain of protein G)	71 residues	MHHHHHHGEN LYFQSMQYKL ILNGKTLKGE TTTEAVDAAT AEKVFKQYAN DNGVDGEWTY DDATKTFTVT E
GFL Peptide	8 residues	YG**GFL**RRI (bold indicates labeled residues)

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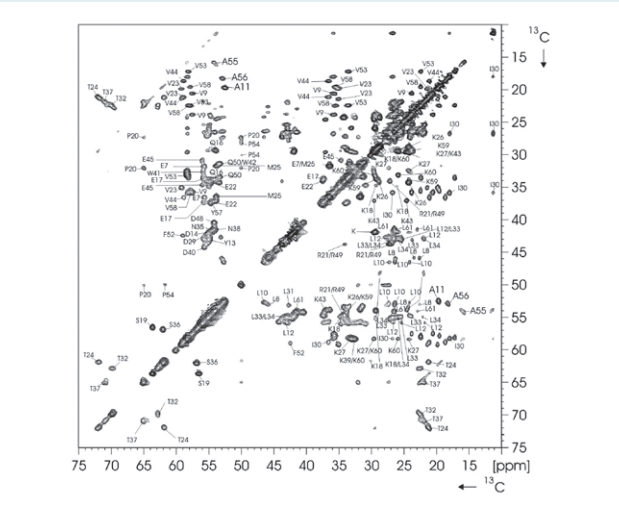
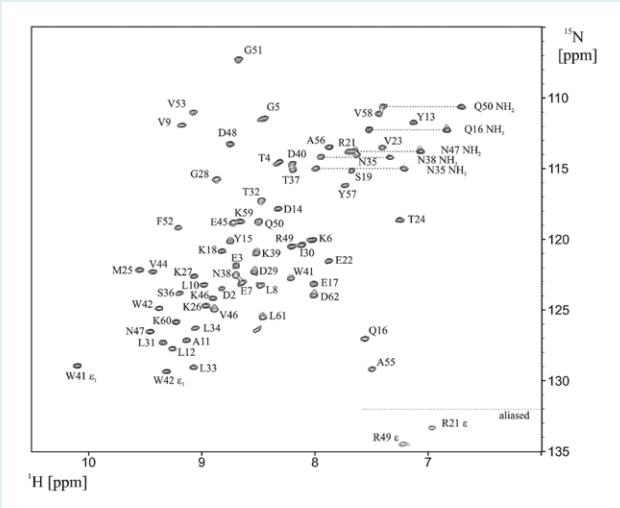
Protein NMR Standards

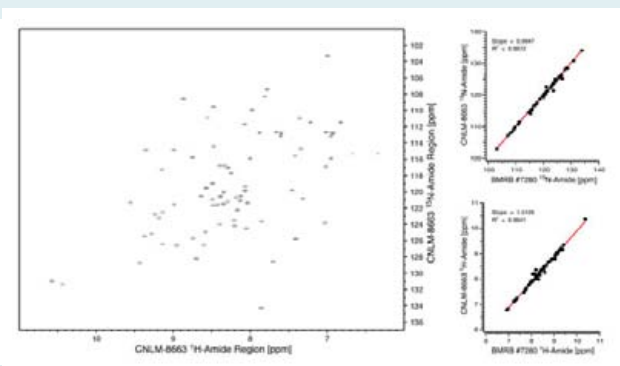


^{13}C-^{13}C solid-state NMR spectrum SH3 Protein (U-^{13}C, U-^{15}N).



^{1}H-^{15}N-HSQC NMR spectrum of SH3 Domain Protein (U-^{13}C, U-^{15}N).

Don't have the time or resources to express your desired protein? Ask your local CIL sales representative about the possibility of having CIL supply you with uniformly labeled, intact protein. Specify the following information: complete protein sequence; type and location of a purification tag (if desired); labeling scheme; level of chemical purity, and desired amount in milligrams.



^{1}H,^{15}N-HSQC of 1.5 mM Immunoglobulin-Binding Domain B1 of Streptococcal Protein G (U-^{13}C, 99%; U-^{15}N, 99%) containing an N-terminal his6-tag and Tobacco Etch Virus protease (TEV) cleavage site, Catalog# CNLM-8663-CA, Lot# 20110209. The ^{15}N-amide (top, right) and ^{1}H-amide (bottom, right) assignments of CNLM-8663-CA show excellent correlation with those previously reported in the Biological Magnetic Resonance Bank for GB1 (BMRB #7280) lacking the his6-TEV leader sequence.

One Letter Designation	Amino Acid	One Letter Designation	Amino Acid	One Letter Designation	Amino Acid
A	Ala	I	Ile	R	Arg
C	Cys	K	Lys	S	Ser
D	Asp	L	Leu	T	Thr
E	Glu	M	Met	V	Val
F	Phe	N	Asn	W	Trp
G	Gly	P	Pro	Y	Tyr
H	His	Q	Gln		

Nucleic Acids

Although solution-state NMR has and will continue to be a powerful and versatile tool for studying the structure and dynamics of RNA and DNA, a recent report shows the promise of using ^{13}C correlation spectroscopy to determine structure of RNA in the solid-state.[1] The different types of information that may be gained from NMR studies of RNA and DNA[2] include the following: Base-pairing patterns; conformational equilibria; site-specific information regarding ligand binding; delineation of secondary structure motifs, such as hairpins and bulges; local structure and dynamics; and global structure derived from residual dipolar couplings.

The most popular approaches to produce labeled RNA molecules for NMR studies use enzymatic *in vitro* transcription methods that employ labeled rNTPs, T7 RNA polymerase and either linearized plasmids or double-stranded DNA as templates. These techniques are used to construct labeled RNA molecules of which all of one type of nucleotide is labeled. Per-deuterated NTPs can be used in combination with protonated NTPs to create RNA molecules in which specific types of nucleotides are protonated, thus allowing spectral editing without the significant signal broadening associated with ^{13}C incorporation.[3]

Because severe signal degeneracy has hampered NMR studies of larger RNAs, key researchers in this area have utilized selectively deuterated rNTPs, in conjunction with *in vitro* synthesis methods, to reduce spectral complexity, spectral line-widths, and for observing NOEs over larger distances.[3]

Labeled DNA oligonucleotides are routinely synthesized using enzymatic *in vitro* methods that utilize labeled dNTPs, a DNA polymerase, and a cDNA template. One advantage of using enzymatic methods over phosphoramidite chemistry is that large oligonucleotides (*e.g.*, >50 nucleotides in length) can be prepared in milligram quantities. Position-specific labeled DNA molecules can be synthesized using standard phosphoramidite chemistry (using CIL's deoxyphosphoramidites) to overcome the limited chemical-shift dispersion of DNA, as well as to create residue-specific probes to obtain functional, structural and dynamic information.

CIL is proud to offer the following selection of labeled mono- and tri-phosphate ribonucleotides, deoxyribonucleotides, and phosphoramidites for use in NMR investigations.

References

1. Cherepanov, A.V.; Glaubitz, C.; Schwalbe, H. **2010**. High-resolution studies of uniformly ^{13}C,^{15}N-labeled RNA by solid-state NMR spectroscopy. *Angewandte Chemi International Ed, 49*, 4747-4750.
2. Furtig, B.; Wohnert, C.; Schwalbe, H. **2003**. *ChemBioChem, 4*, 936-962.
3. Lu, K.; Miyazaki, Y.; Summers, M. **2010**. Isotope labeling strategies for NMR studies of RNA. *J Biomol NMR, 46*, 113-125.

Cassia LLC was founded in 2005 by noted NMR spectroscopist Dr. Jamie Williamson and Dr. Lincoln Scott. CIL and Cassia have a special relationship which makes use of CIL's isotopic material production and marketing and Cassia's special knowledge of RNA and DNA biosynthesis. Since 2005, CIL and Cassia have developed the most extensive product line of stable isotope-labeled RNA and DNA triphosphates, DNA phosphoramidites and other related compounds. All of these products are routinely available for immediate shipment from stock at CIL.

Top 10 Reasons to Use Ammonium Salts

1. Self-buffering (pH ~ 7.6)
2. "Soft Cation"
3. Nucleotides of ammonium salts are active with polymerases, synthetases, and phosphatases.
4. Volatile counter-ion
5. The ammonium cation can be easily exchanged using DOWEX cation exchange resin
6. The pH does not change during drying of the nucleotide (*i.e.* "speed-vac," lyophilize)
7. Stoichiometry between the counter-ion and the nucleotide is preserved
8. Routinely compatible in down-stream syntheses
9. Compatible in a variety of down-stream chromatography applications
10. Tested to be comparable in side-by-side *in vitro* transcription reactions!

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Nucleic Acids

rNTPs (^{15}N) (^{13}C,^{15}N)

Catalog No.	Description
NLM-3987-CA	Adenosine 5′-triphosphate, ammonium salt (U-$^{15}N_5$; 98-99%) (CP >90%) (in soln)
NLM-4266-CA	Cytidine 5′-triphosphate, ammonium salt (U-$^{15}N_3$; >96%) (CP >90%) (in soln)
NLM-4268-CA	Guanosine 5′-triphosphate, ammonium salt (U-$^{15}N_5$; 98-99%) (CP >90%) (in soln)
NLM-4270-CA	Uridine 5′-triphosphate, ammonium salt (U-$^{15}N_2$; 98-99%) (CP >90%) (in soln)
NLM-3987	Adenosine 5′-triphosphate, lithium salt (U-$^{15}N_5$, 98%) (CP >90%) (in soln)
NLM-4266	Adenosine 5′-triphosphate, lithium salt (U-$^{15}N_3$, 98%) (CP >90%) (in soln)
NLM-4268	Guanosine 5′-triphosphate, lithium salt (U-$^{15}N_5$, 98%) (CP >90%) (in soln)
NLM-4270	Uridine 5′-triphosphate, lithium salt (U-$^{15}N_2$, 98%) (CP >90%) (in soln)
NLM-7519	Set of 4 Ribonucleoside 5′-triphosphates (U-^{15}N, 98%) (CP >90%) (lithium salt/in soln)
CNLM-4265-CA	Adenosine 5′-triphosphate, ammonium salt (U-^{13}C, U-^{15}N; 98-99%) (CP >90%) (in soln)
CNLM-4267-CA	Cytidine 5′-triphosphate, ammonium salt (U-^{13}C, U-^{15}N; 96-98%) (CP >90%) (in soln)
CNLM-4269-CA	Guanosine 5′-triphosphate, ammonium salt (U-^{13}C, U-^{15}N; 98-99%) (CP >90%) (in soln)
CNLM-4271-CA	Uridine 5′-triphosphate, ammonium salt (U-^{13}C, U-^{15}N; 98-99%) (CP >90%) (in soln)
CNLM-7503-CA	Set of 4 Ribonucleoside 5′-triphosphates (U-^{13}C, U-^{15}N; 98-99%) (CP >90%) (ammonium salt/in soln)
CNLM-4265	Adenosine 5′-triphosphate, lithium salt (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 98%) (CP >90%) (in soln)
CNLM-4267	Cytidine 5′-triphosphate, lithium salt (U-$^{13}C_9$, 98%; U-$^{15}N_3$, 98%) (CP >90%) (in soln)
CNLM-4269	Guanosine 5′-triphosphate, lithium salt (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 98%) (CP >90%) (in soln)
CNLM-4271	Uridine 5′-triphosphate, lithium salt (U-$^{13}C_9$, 98%; U-$^{15}N_2$, 98%) (CP >90%) (in soln)
CNLM-7503	Set of 4 Ribonucleoside 5′-triphosphates (U-^{13}C, 98%; U-^{15}N, 98%) (CP >90%) (lithium salt/in soln)

rNTPs (D)

DLM-7514-CA	Adenosine 5′-triphosphate, ammonium salt (U-D_8, 97%+) (CP >90%) (in soln)
DLM-7515-CA	Cytidine 5′-triphosphate, ammonium salt (U-D_8, 97%+) (CP >90%) (in soln)
DLM-7516-CA	Guanosine 5′-triphosphate, ammonium salt (U-D_7, 97%+) (CP >90%) (in soln)
DLM-7517-CA	Uridine 5′-triphosphate, ammonium salt (U-D_8, 97%+) (CP >90%)
DLM-7514	Adenosine 5′-triphosphate, lithium salt (U-D_8, 97%+) (CP >90%) (in soln)
DLM-7515	Cytidine 5′-triphosphate, lithium salt (U-D_8, 97%+) (CP >90%) (in soln)
DLM-7516	Guanosine 5′-triphosphate, lithium salt (U-D_7, 97%+) (CP >90%) (in soln)
DLM-7517	Uridine 5′-triphosphate, lithium salt (U-D_8, 97%+) (CP >90%) (in soln)
DLM-7518	Set of 4 Ribonucleoside 5′-triphosphates (U-D, 98%) (CP >90%) (lithium salt/in soln)

"Without these labeled rNTPs from CIL we would not have been able to prepare such high quality samples which made the assignment and structure determination possible."

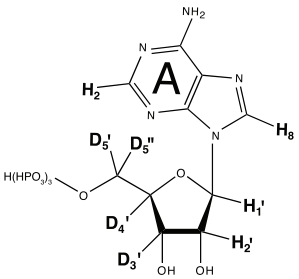
Dr. Michael Durney
Department of Molecular and Cellular Biology
Harvard University

(continued)

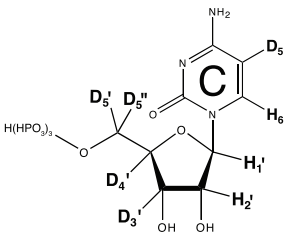
Nucleic Acids

rNTPs (Selectively Labeled D)

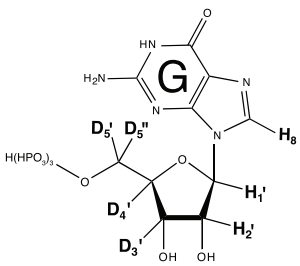
Catalog No.	Description
DLM-7862	Equimolar Mix: ATP, GTP, (ribose-3′,4′,5′,5″-D_4, 98%); CTP, UTP (5-D_1, ribose-3′,4′,5′,5″-D_4, 98%) NH_4^+ salt (see structures below)
DLM-8815-CA	Adenosine 5′-triphosphate, ammonium salt (2-D, 97%) (CP >90%) (in soln)
DLM-8594-CA	Cytidine 5′-triphosphate, ammonium salt (cytosine-5-D, 6-H; ribose-1,2,3,4,5,5-D_6, 96-97%)
NLM-8637-CA	Uridine 5′-triphosphate, ammonium salt (uracil-5-D, 6-H; ribose-1,2,3,4,5,5-D_6, 96-97%)



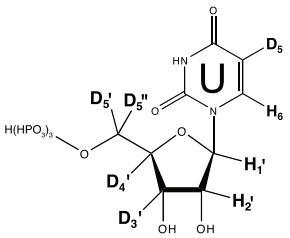
ribose-3′,4′,5′,5″-d_4, 98%



5-d_1; ribose-3′,4′,5′,5″-d_4, 98%

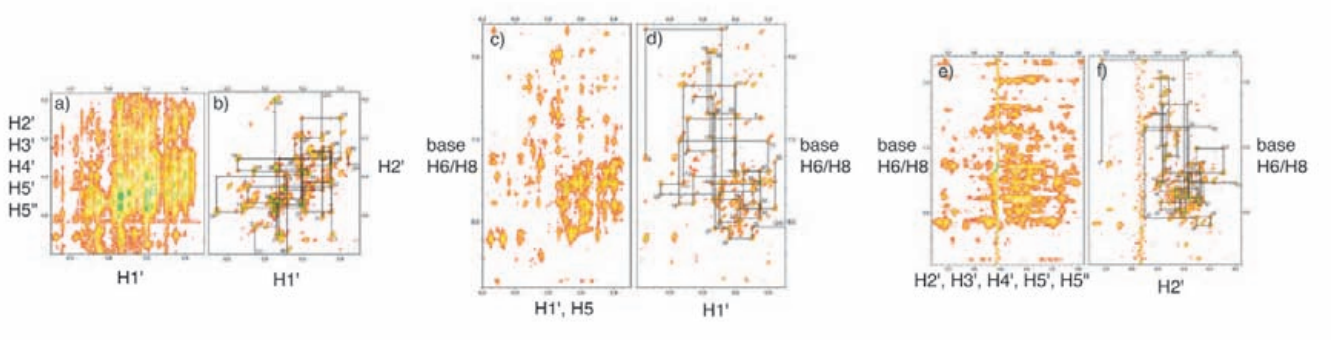


ribose-3′,4′,5′,5″-d_4, 98%



5-d_1; ribose-3′,4′,5′,5″-d_4, 98%

DLM-8922-CA	Adenine-5′-triphosphate, ammonium salt (ribose-3′,4′,5′,5″-D_4, 98%)
DLM-8924-CA	Cytidine-5′-triphosphate, ammonium salt (5-D_1, ribose-3′,4′,5′,5″-D_4, 98%)
DLM-8923-CA	Guanosine-5′-triphosphate, ammonium salt (ribose-3′,4′,5′,5″-D_4, 98%)
NLM-8925-CA	Uridine-5′-triphosphate, ammonium salt (5-D_1, ribose-3′,4′,5′,5″-D_4, 98%)



1H-1H-NOESY Spectra of the Tetraloop-Receptor RNA (45 nt dimer; 30 kDa). Spectra above are from unlabeled RNA (a,c,e) and selectively deuterated RNA (b,d,f). The selectively deuterated RNA was prepared using the equimolar mix, CIL Catalog # DLM-7862. The left two panels (a,b) contain NOEs between the H1′ proton and all other ribose protons. The middle two panels (c,d) contain NOEs between the base protons and H1′ protons. The right two panels (e,f) contain NOEs between the base and other ribose protons. Spectra taken from Davis *et al*. (2005) were provided courtesy of Prof. Sam Butcher at the University of Wisconsin. The sequential assignment pattern of inter and intra nucleotide NOEs is shown for the D_5-RNA. The advantages of the selectively deuterated pattern are evident in these key regions of the spectra.

Reference

Davis, J.H.; Tonelli, M.; Scott, L.G.; Jaeger, L.; Williamson, J.R.; Butcher, S. **2005**. Helical packing in solution: NMR structure of a 30 kDa GAAA tetraloop-receptor complex. *J Mol Biol, 352,* 371-382.

Nucleic Acids

rNMPs (^{15}N) (^{13}C,^{15}N)

Catalog No.	Description
NLM-3792	Adenosine 5′-monophosphate (U-$^{15}N_5$, 96-98%)
NLM-3793	Cytidine 5′-monophosphate (U-$^{15}N_3$, 96-98%)
NLM-3794	Guanosine 5′-monophosphate (U-$^{15}N_5$, 96-98%)
NLM-3795	Uridine 5′-monophosphate (U-$^{15}N_2$, 96-98%)
CNLM-3802	Adenosine 5′-monophosphate (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 96-98%)
CNLM-3803	Cytidine 5′-monophosphate (U-$^{13}C_9$, 98%; U-$^{15}N_3$, 96-98%)
CNLM-3804	Guanosine 5′-monophosphate (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 96-98%)
CNLM-3805	Uridine 5′-monophosphate (U-$^{13}C_9$, 98%; U-$^{15}N_2$, 96-98%)

dNTPs (^{15}N) (^{13}C,^{15}N)

NLM-6217-CA	2′-Deoxyguanosine 5′-triphosphate, ammonium salt (U-$^{15}N_5$; 98-99%) (CP >90%) (in soln)
NLM-6215	2′-Deoxyadenosine 5′-triphosphate, lithium salt (U-$^{15}N_5$, 98%) (CP >90%) (in soln)
NLM-6216	2′-Deoxycytidine 5′-triphosphate, lithium salt (U-$^{15}N_3$, 98%) (CP >90%) (in soln)
NLM-6217	2′-Deoxyguanosine 5′-triphosphate, lithium salt (U-$^{15}N_5$, 98%) (CP >90%) (in soln)
NLM-6218	Thymidine 5′-triphosphate, lithium salt (U-$^{15}N_2$, 98%) (CP >90%) (in soln)
NLM-7512	Set of 4 2′-Deoxyribonucleoside 5′-triphosphates (U-^{15}N, 98%) (CP >90%) (lithium salt/in soln)
CNLM-6219-CA	2′-Deoxyadenosine 5′-triphosphate (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 97-98%) (CP >90%)
CNLM-6221-CA	2′-Deoxyguanosine 5′-triphosphate (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 96-98%) (CP >90%)
CNLM-6219	2′-Deoxyadenosine 5′-triphosphate, lithium salt (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 98%) (CP >90%) (in soln)
CNLM-6220	2′-Deoxycytidine 5′-triphosphate, lithium salt (U-$^{13}C_9$, 98%; U-$^{15}N_3$, 98%) (CP >90%) (in soln)
CNLM-6221	2′-Deoxyguanosine 5′-triphosphate, lithium salt (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 98%) (CP >90%) (in soln)
CNLM-6222	Thymidine 5′-triphosphate, lithium salt (U-$^{13}C_{10}$, 98%; U-$^{15}N_2$, 98%) (CP >90%) (in soln)
CNLM-7513	Set of 4 2′-Deoxyribonucleoside 5′-triphosphates (U-^{13}C, 98%; U-^{15}N, 98%) (CP >90%) (lithium salt/in soln)

dNTPs (D)

DLM-7507	2-Deoxyadenosine 5′-triphosphate, lithium salt (U-D, 97%+) (CP >90%) (in soln)
DLM-7508	2-Deoxycytidine 5′-triphosphate, lithium salt (U-D, 97%+) (CP >90%) (in soln)
DLM-7509	2-Deoxyguanosine 5′-triphosphate, lithium salt (U-D, 97%+) (CP >90%) (in soln)
DLM-7510	Thymidine 5′-triphosphate, lithium salt (U-D, 97%+) (CP >90%) (in soln)
DLM-7511	Set of 4 2-Deoxyribonucleoside 5′-triphosphates (U-D, 98%) (CP >90%) (lithium salt/in soln)

dNMPs (^{15}N) (^{13}C,^{15}N)

NLM-3919	2′-Deoxyadenosine 5′-monophosphate (U-$^{15}N_5$, 98%)
NLM-3921	2′-Deoxycytidine 5′-monophosphate (U-$^{15}N_3$, 96%)
NLM-6835	2′-Deoxyguanosine 5′-monophosphate (U-^{15}N, 98%)
NLM-3925	Thymidine 5′-monophosphate (U-$^{15}N_2$, 98%)
CNLM-3918	2′-Deoxyadenosine 5′-monophosphate, lithium salt (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 98%)
CNLM-6834	2′-Deoxycytidine 5′-monophosphate (U-^{13}C, 98%; U-^{15}N, 98%)
CNLM-6836	2′-Deoxyguanosine 5′-monophosphate, lithium salt (U-^{13}C, 98%; U-^{15}N, 98%)
CNLM-3924	Thymidine 5′-monophosphate (U-$^{13}C_{10}$, 98%; U-$^{15}N_2$, 98%)
CNLM-7871	Set of 4 2′-Deoxyribonucleoside 5′-monophosphates (U-^{13}C, 98%; U-^{15}N, 98%) (in soln)

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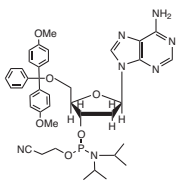
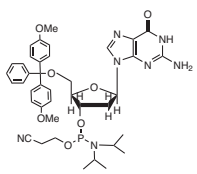
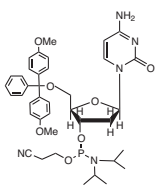
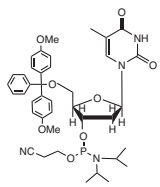
(continued)

Nucleic Acids

Deoxyphosphoramidites (^{13}C, ^{15}N)

Catalog No.	Description
CNLM-6828	2′-Deoxyadenosine phosphoramidite (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 98%) (CP 95%)
CNLM-6830	2′-Deoxycytidine phosphoramidite (U-$^{13}C_9$, 98%; U-$^{15}N_3$, 98%) (CP 95%)
CNLM-6825	2′-Deoxyguanosine phosphoramidite (U-$^{13}C_{10}$, 98%; U-$^{15}N_5$, 98%) (CP 95%)
CNLM-6824	Thymidine phosphoramidite (U-$^{13}C_{10}$, 98%; U-$^{15}N_2$, 98%) (CP 95%)

Deoxyphosphoramidites (^{15}N)

Catalog No.	Description		Catalog No.	Description	
NLM-6829	2′-Deoxyadenosine phosphoramidite (U-$^{15}N_5$, 98%) (CP 95%)		NLM-6826	2′-Deoxyguanosine phosphoramidite (U-$^{15}N_5$, 98%) (CP 95%)	
NLM-6827	2′-Deoxycytidine phosphoramidite (U-$^{15}N_3$, 98%) (CP 95%)		NLM-6823	Thymidine phosphoramidite (U-$^{15}N_2$, 96-98%) (CP 95%)	

"We've been using CIL's perdeuterated rNTPs for many years to facilitate NMR studies of larger RNAs. Their newer products, which include partially deuterated rNTPs, have been extraordinarily helpful, and have enabled high resolution NMR structural studies of RNAs that were previously intractable."

Dr. Michael Summers
Howard Hughes Medical Institute
University of Maryland, Baltimore County

"When my lab makes labeled RNA, we count on high yields and purity. We have been using $^{13}C/^{15}N$ ribonucleotide triphosphates (rNTPs) and selectively deuterated rNTPs from CIL for the past 10 years. We have always been very happy with the performance and quality of the CIL rNTPs. They are the gold standard."

Dr. Samuel Butcher
Department of Biochemistry
University of Wisconsin-Madison

Miscellaneous RNA and DNA Products

NLM-6924	Adenine•HCL ($^{15}N_5$, 98%)
CLM-1654	Adenine (8-^{13}C, 95%)
CLM-3605	Adenosine•H_2O (ribose-1-^{13}C, 99%) (CP 95%)
CLM-3678	Adenosine (ribose-$^{13}C_5$, 98%+) (CP 97%)
NLM-3895	2′-Deoxyadenosine (U-$^{15}N_5$, 96-98%) (see structure above)
NLM-3797	Cytidine (U-$^{15}N_3$, 96-98%)
CNLM-3807	Cytidine (U-$^{13}C_9$, 98%; U-$^{15}N_3$, 96-98%)
NLM-3897	2′-Deoxycytidine (U-$^{15}N_3$, 96-98%) (see structure above)
CLM-1019	Guanine (8-^{13}C, 98%)
CNLM-3990	Guanine (8-^{13}C, 98%; 7,9-$^{15}N_2$, 98%)
NLM-3798	Guanosine (U-$^{15}N_5$, 96-98%)
NLM-3899-CA	2′-Deoxyguanosine•H_2O (U-$^{15}N_5$, 98%) (CP 95%+) (see structure above)
CLM-3764	Thymine (6-^{13}C, 99%)
DLM-1089	Thymine (α,α,α,6-D_4, 98%)
NLM-3995	Thymine (1,3-$^{15}N_2$, 98%)
CNLM-6945	Thymine (U-$^{13}C_5$, 98%; U-$^{15}N_2$, 98%)
CLM-3647	Thymidine (methyl-^{13}C, 98%)
DLM-3327	Thymidine (methyl-D_3, ring-6-D_1, 97%+) (CP 95%) (see structure above)
NLM-3901	Thymidine (U-$^{15}N_2$, 96-98%) (CP 97%)
CNLM-3902	Thymidine (U-$^{13}C_{10}$, 98%; U-$^{15}N_2$, 96-98%)

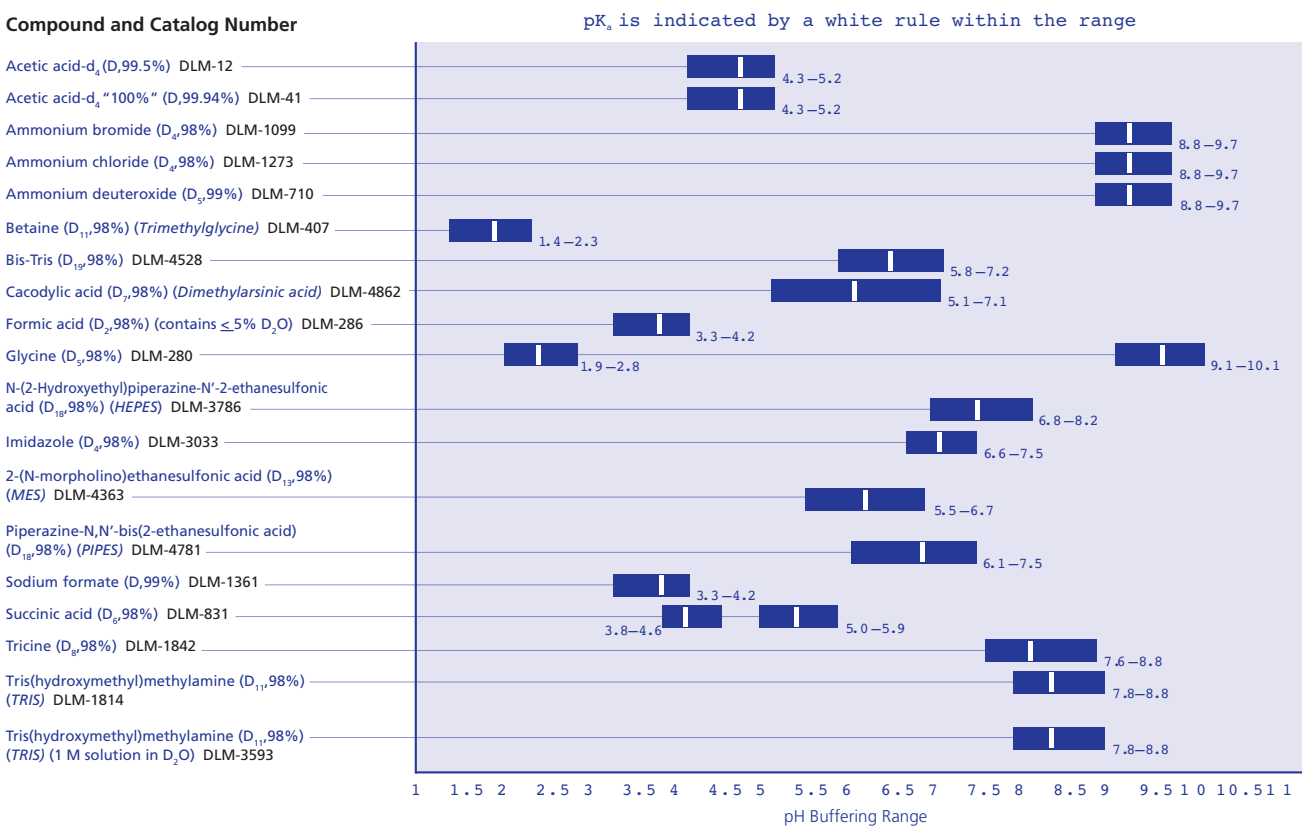
Deuterated Buffers

CIL offers the following wide selection of deuterated buffers for use with aqueous solutions:

Catalog No.	Description
DLM-12	Acetic acid-D_4 (D, 99.5%)
DLM-41	Acetic acid-D_4 "100%" (D, 99.93%)
DLM-1099	Ammonium bromide (D_4, 98%)
DLM-1273	Ammonium chloride (D_4, 98%)
DLM-710	Ammonium deuteroxide (D_5, 99%) (~25% in soln D_2O)
DLM-407	Betaine (D_{11}, 98%)
DLM-4528	Bis-Tris (D_{19}, 98%)
DLM-4862	Cacodylic acid (D_7, 98%)
DLM-286	Formic acid (D_2, 98%) (<5% D_2O)
DLM-280	Glycine (D_5, 98%)

Catalog No.	Description
DLM-3786	Hepes (D_{18}, 98%)
DLM-3033	Imidazole (D_4, 98%)
DLM-4363	Mes (D_{13}, 98%)
DLM-4781	Pipes (D_{18}, 98%)
DLM-1361	Sodium formate (D, 98%)
DLM-831	Succinic acid (D_6, 98%)
DLM-1842	Tricine (D_8, 98%)
DLM-4779	Trimethylamine *N*-oxide (D_9, 98%)
DLM-1814	TRIS (D_{11}, 98%)
DLM-3593	TRIS (D_{11}, 98%) 1 M in D_2O

pH Buffering Range Chart



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Application Note 12

Optimization of BioExpress® 1000 Supplementation of M9 Cultures

Marwa Rhima, Lori C. Neil and Kevin H. Gardner
Department of Biochemistry, UT Southwestern Medical Center at Dallas

Application Note 14

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

André Strauss, Gabriele Fendrich and Wolfgang Jahnke
Novartis Institutes for Biomedical Research, Basel, Switzerland

Application Note 15

Top Ten Tips for Producing $^{13}C,^{15}N$ Protein in Abundance

Deborah A. Berthold, Victoria J. Jeisy, Terry L. Sasser, John J. Shea, Heather L. Frericks, Gautam Shah, and Chad M. Rienstra
Departments of Chemistry and Biochemistry, University of Illinois at Urbana-Champaign

Application Note 16

Specific Isotope Labeling of Methyl Groups Has Extended the Molecular Weight Limits for NMR Studies of Protein Structure and Dynamics

John B. Jordan[1] and Richard W. Kriwacki[1,2]
[1]St. Jude Children's Research Hospital, Department of Structural Biology
[2]Department of Molecular Sciences, University of Tennessee Health Sciences Center

Application Note 20

Effective Site-Specific Isotopic Labeling ($^{13}C,^{15}N$ Glycine; $^{13}C,^{15}N$ Phenylalanine; ^{15}N Tryptophan) Expression Optimization Using BioExpress® 2000 Media

Kenneth C. Bonanno
Vertex Pharmaceuticals, Inc., 130 Waverly Street, Cambridge, MA 02139

Application Note 22

[2,3-$^{13}C_2$]-Labeled Aromatic Residues as a Means to Improving Signal Intensities and Kick-Starting the Assignment of Membrane Proteins by Solid-State MAS-NMR

†Matthias Hiller,† Victoria A. Higman,† Stefan Jehle,† Barth-Jan van Rossum,† Werner Kühlbrandt‡ and Hartmut Oschkinat†
†Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Strasse 10, 13125 Berlin, Germany, and ‡Max-Planck-Institut für Biophysik, Max-von-Laue-Str. 3, 60438 Frankfurt am Main, Germany

Application Note 25

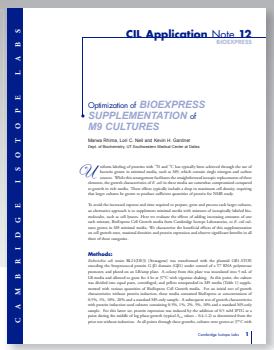
Isotope Labeling of Alanine Methyl Groups on a Deuterated Background for NMR Studies of High-Molecular-Weight Proteins

Chenyun Guo, Raquel Godoy-Ruiz and Vitali Tugarinov
Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742

Application Note 26

Uniform Isotope Labeling of Eukaryotic Proteins in Methylotrophic Yeast for High-Resolution NMR Studies – Extension to Membrane Proteins

Ying Fan, Lichi Shi, Vladimir Ladizhansky and Leonid S. Brown
Departments of Physics and Biophysics Interdepartmental Group, University of Guelph
50 Stone Road East, Guelph, Ontario, N1G 2W1, Canada



Optimization of BioExpress® 1000 Supplementation of M9 Cultures

Marwa Rhima, Lori C. Neil and Kevin H. Gardner
Department of Biochemistry
UT Southwestern Medical Center at Dallas

Uniform labeling of proteins with ^{15}N and ^{13}C has typically been achieved through the use of bacteria grown in minimal media, such as M9, which contain single nitrogen and carbon sources. While this arrangement facilitates the straightforward isotopic replacements of these elements, the growth characteristics of *E. coli* in these media are somewhat compromised compared to growth in rich media. These effects typically include a drop in maximum cell density, requiring that larger cultures be grown to produce sufficient quantities of protein for NMR study.

To avoid the increased expense and time required to prepare, grow and process such larger cultures, an alternative approach is to supplement minimal media with mixtures of isotopically labeled biomolecules, such as cell lysates. Here we evaluate the effects of adding increasing amounts of one such mixture, BioExpress® 1000 Cell Growth media from Cambridge Isotope Laboratories, to *E. coli* cultures grown in M9 minimal media. We characterize the beneficial effects of this supplementation on cell growth rates, maximal densities and protein expression and observe significant benefits in all three of these categories.

Methods

Escherichia coli strain BL21(DE3) (Stratagene) was transformed with the plasmid GB1-STOP, encoding the Streptococcal protein GB1 domain (GB1) under control of a T7 RNA polymerase promoter, and plated on an LB/amp plate. A colony from this plate was inoculated into 5 mL of LB media and allowed to grow for four hours at 37°C with vigorous shaking. At this point, the culture was divided into equal parts, centrifuged, and pellets resuspended in M9 media (Table 1) supplemented with various quantities of BioExpress® 1000 Cell Growth media. For an initial test of growth characteristics without protein induction, these media contained BioExpress® 1000 at concentrations of 0.5%, 1%, 10%, 20% and a standard M9-only sample. A subsequent test of growth characteristics with protein induction used cultures containing 0.5%, 1%, 2%, 5%, 10% and a standard M9-only sample. For this latter set, protein expression was induced by the addition of 0.5 mM IPTG at a point during the middle of log phase growth (typical A_{600} values ~ 0.4-1.2) as determined from the prior test without induction. At all points through these growths, cultures were grown at 37°C with vigorous shaking and densities were monitored by turbidity at 600 nm (A_{600}). Induced cultures were harvested three hours post-induction, at which point they were analyzed for protein expression (SDS-PAGE) and total cell mass (wet weight).

Table 1: M9 minimal media

Na_2HPO_4	6 g/L	$MgSO_4 \bullet 7H_2O$	246 mg/L (1 mM)
KH_2PO_4	3 g/L	$CaCl_2 \bullet 2H_2O$	14.7 mg/L (0.1 mM)
NaCl	0.5 g/L	thiamine	10 mg/L
NH_4Cl	1 g/L	biotin	10 mg/L
glucose	3 g/L	ampicillin	100 mg/L

Results

Supplementing standard M9 media with BioExpress® 1000 Cell Growth media provided several significant benefits, including decreased doubling times and increased total cell mass. These favorable characteristics were observed in cultures supplemented with BioExpress® 1000 at amounts significantly below the recommended working level of 1% v/v. In detail:

- Supplementing M9 media with BioExpress® 1000 significantly shortened doubling times by up to 40%. As detailed in Table 2, BioExpress® 1000-supplemented M9 cultures had doubling times between 31-49 minutes, as compared to 57 minutes for a non-supplemented culture. The degree of doubling time shortening correlated with the amount of added BioExpress® 1000, with the most pronounced effects observed at concentrations of at least 1%.

Table 2: Effect of BioExpress® 1000 supplementation on doubling times, cell densities

media	doubling time (min)	max A_{600} (non-induced)	A_{600} (3 hour post induction)
Control (M9)	57.3 min	2.20	0.92
M9 + 0.5% v/v BioExpress® 1000	48.7 min	2.86	1.75
M9 + 1%	38.2 min	3.09	1.63
M9 + 2%	34.7 min	n.d.	1.97
M9 + 5%	33.3 min	n.d.	1.90
M9 + 10%	31.6 min	5.26	2.81

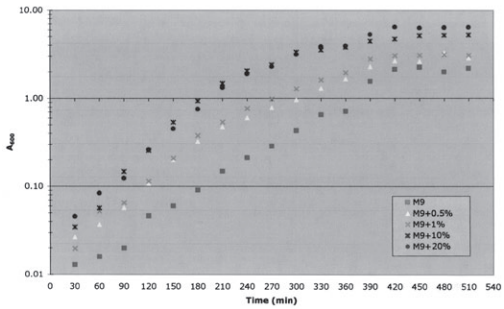
- Adding BioExpress® 1000 significantly increased the maximum density obtained from these cultures, regardless of whether protein expression was induced or not. As shown in Table 2 and Figure 1, supplementation with BioExpress® 1000 at levels as low as 0.5% v/v increased post-induction densities by factors of 2-3 fold compared to M9 controls.

- BioExpress® 1000-supplemented cultures also expressed higher amounts of the induced GB1 protein on a per-cell basis, as shown in the SDS-PAGE analyses presented in Figure 2. Importantly, this effect is specific to the induced protein and does not represent an increase in the levels of all proteins, as shown by the equivalent staining of non-induced proteins across all of the samples in Figure 2.

Summary

The supplementation of standard M9 media with complex mixtures of biomolecules and their precursors, such as BioExpress® 1000 Cell Growth media, offers a route to straightforwardly improve the yield of uniformly labeled protein from bacterial cultures. As shown here, this supplementation can improve three key aspects of this process: growth rate, maximal cell density, and the degree of overexpression of a recombinant protein. Significant improvements in each of these can be obtained by supplementing with modest (1% v/v) amounts of BioExpress® 1000, with further improvement possible at higher levels.

BioExpress® 1000 Growth Media Evaluation:
Non-induced samples



BioExpress® 1000 Growth Media Evaluation:
Induced samples

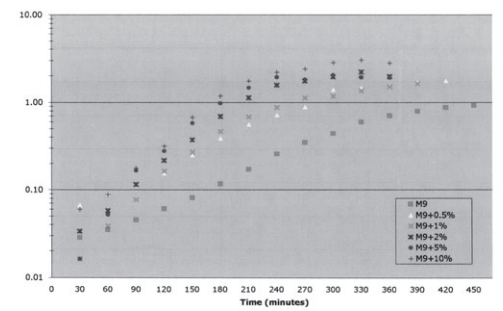


Figure 1. Growth curves of BL21(DE3) cells containing pGB1-STOP in M9 media supplemented by various concentrations of BioExpress® 1000 Cell Growth Media. Protein expression in the induced cultures was triggered by the addition of 0.5mM IPTG at mid-log phase points that ranged from A600~0.4 (M9) to A600~1.2 (M9+10% BioExpress® 1000).

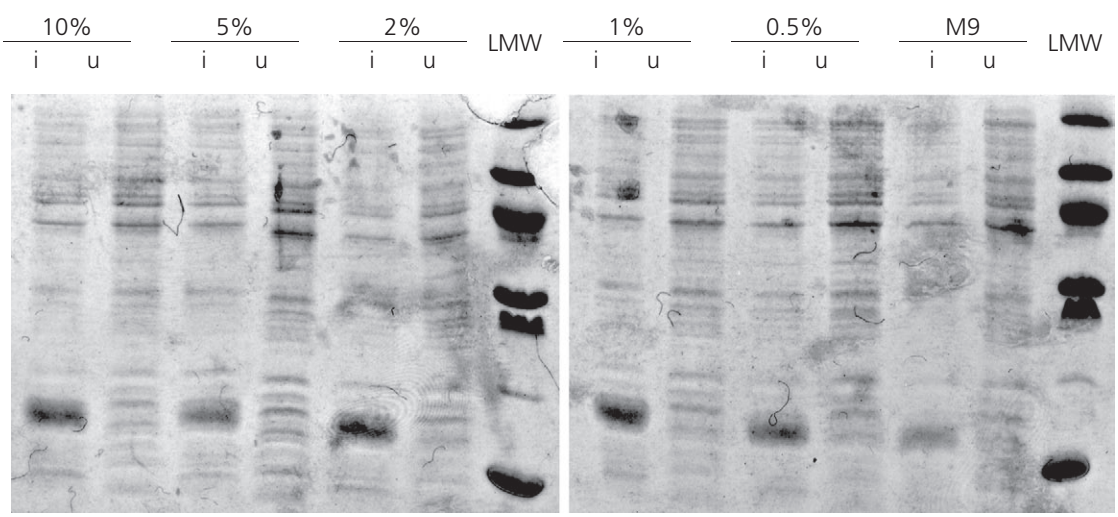
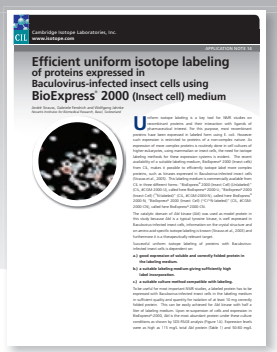


Figure 2. SDS-PAGE analysis of whole cells from non-induced and induced cultures of cells grown in M9 media containing varying concentrations of BioExpress® 1000 Cell Growth media. Both uninduced (u) and induced (i) samples of cultures grown with each concentration of BioExpress® 1000 were evaluated as identified by the percentage supplementation of BioExpress® 1000. All samples were corrected for cell density prior to loading. The band corresponding to GB1 is identified with an arrow on the left side of the figure. LMW: low molecular weight markers.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APPLICATION NOTE 14
Efficient uniform isotope labeling of proteins expressed in Baculovirus-infected insect cells using BioExpress® 2000 (Insect cell) medium

Efficient Uniform Isotope 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 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 Catalog # CGM-2000-U), called here BioExpress® 2000-U; "BioExpress® 2000 (Insect Cell) (^{15}N-labeled)" (CIL Catalog # CGM-2000-N), called here BioExpress® 2000-N; "BioExpress® 2000 (Insect Cell) (^{13}C,^{15}N-labeled)" (CIL Catalog # 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:

a) good expression of soluble and correctly folded protein in the labeling medium.
b) a suitable labeling medium giving sufficiently high label incorporation.
c) 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 resuspension of 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 µM 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 medium (BioExpress® 2000-N), and the ^{13}C,^{15}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}C,^{15}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}C,^{15}N-labeled Abl, high quality ^{1}H-^{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 ^{1}H-^{15}N-HSQC spectrum of ^{15}N-labeled Abl (Figure 2). The same is true for the ^{1}H-^{15}N-HSQC spectrum of ^{13}C,^{15}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

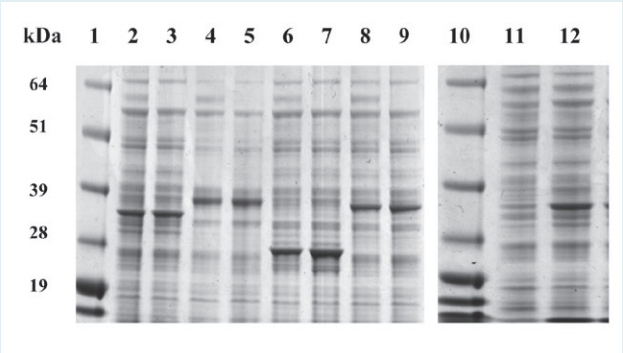


Figure 1. SDS-PAGE analysis by Coomassie blue staining of total lysates from BV-infected Sf9 cells expressing recombinant. proteins, cultured in 50 mL shake flask cultures. Lanes 1 and 10 show molecular weight marker proteins (Invitrogen, SeeBlue Plus2).

A: Expression of Abl kinase (6His-TEV-GAMDP-Abl/S^{229}-S^{500}) in presence of 16 µM STI571(lanes 2 and 3), KDR kinase (M^{806}-K^{941}/D50/P^{992}-D^{1171}-ThrcS-6His) in presence of 10 µM PTK^{787} (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 µM 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 (x10^6c/mL)	Viability (%)	Fresh weight (g/30 mL)	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.

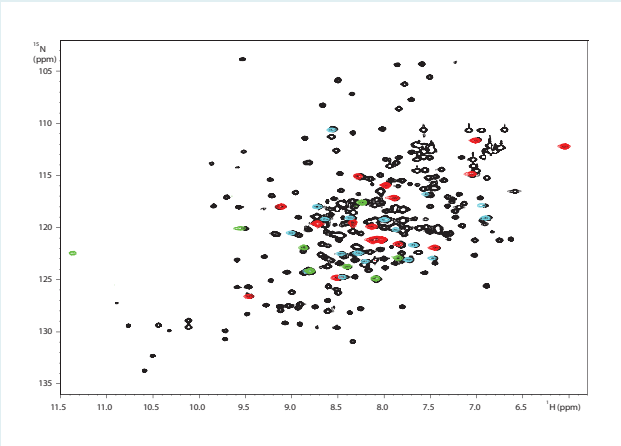


Figure 2. [^{15}N;^{1}H]-HSQC spectrum for uniformly ^{15}N-labeled Abl kinase (in black) expressed in BV-infected Sf9 cells, superimposed with the resonances from Abl kinase labeled with ^{15}N-Phe (in green), ^{15}N-Tyr (in red), ^{15}N-Val (in blue) (from Strauss *et al*., 2003). One resonance at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

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

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.

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 ~ 1.5 × 10^6 c/mL (~ 3 days), sterile centrifuge the cells at 400G for 20 minutes at 20°C.
5 Resuspend 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 × 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 3 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); resuspend 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 purifiy 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.

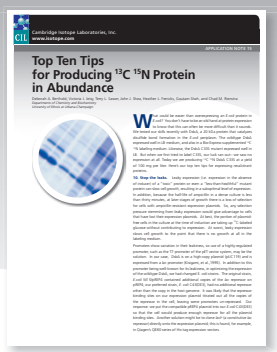
Figure 4. Prerequisite for successful uniform istope labeling in a protein with BV-infected insect cells.

References

Strauss, A.; Bitsch, F.; Cutting, B.; Fendrich, F.; Graff, P.; Liebetanz, J.; Zurini, M.; and Jahnke, W. **2003**. *J Biomol NMR*, *26*, 367-372.

Strauss, A.; Bitsch, F.; Fendrich, F.; Graff, P.; Knecht, R.; Meyhack, B.; and Jahnke, W. **2005**. *J Biomol NMR*, *31*, 343-349.

O'Reilly, D.R.; Miller, L.K.; and Luckow, V.A. **1994**. *Baculovirus Expression Vectors*, Oxford University Press, New York, NY.



Top Ten Tips for Producing $^{13}C,^{15}N$ Protein in Abundance

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What could be easier than overexpressing an *E.coli* protein in *E.coli*? You don't have to be an old hand at protein expression to know that this can often be more difficult than it sounds. We tested our skills recently with DsbA, a 20 kDa protein that catalyzes disulfide bond formation in the *E.coli* periplasm. The wildtype DsbA expressed well in LB medium, and also in a BioExpress® 1000-supplemented $^{13}C,^{15}N$ labeling medium. Likewise, the DsbA C33S mutant expressed well in LB. But when we first tried to label C33S, our luck ran out – we saw no expression at all. Today we are producing $^{13}C,^{15}N$ DsbA C33S at a yield of 100 mg per liter. Here's our top ten tips for expressing recalcitrant proteins.

10. Stop the leaks. Leaky expression (*i.e.* expression in the absence of inducer) of a "toxic" protein or even a "less-than-healthful" mutant protein can slow cell growth, resulting in a suboptimal level of expression. In addition, because the half-life of ampicillin in a dense culture is less than 30 minutes, at later stages of growth there is a loss of selection for cells with ampicillin-resistant expression plasmids. So, any selection pressure stemming from leaky expression would give advantage to cells that have lost their expression plasmids. At best, the portion of plasmid-free cells in the culture at the time of induction are taking up ^{13}C-labeled glucose without contributing to expression. At worst, leaky expression slows cell growth to the point that there is no growth at all in the labeling medium.

Promoters show variation in their leakiness, so use of a highly regulated promoter, such as the T7 promoter of the pET vector system, may be the solution. In our case, DsbA is on a high-copy plasmid (pUC119) and is expressed from a *lac* promoter (Kisigami, *et al.*,1995). In addition to this promoter being well-known for its leakiness, in optimizing the expression of the wildtype DsbA, we had changed *E. coli* strains. The original strain, *E.coli* M15/pREP4 contained additional copies of the *lac* repressor on pREP4; our preferred strain, *E. coli* C43(DE3), had no additional repressor other than the copy in the host genome. It was likely that the repressor binding sites on our expression plasmid titrated out all the copies of the repressor in the cell, leaving some promoters unrepressed. Our response: we put the compatible pREP4 plasmid into our *E.coli* C43(DE3) so that the cell would produce enough repressor for all the plasmid binding sites. Another solution might be to clone *lacIq* (a constitutive *lac* repressor) directly onto the expression plasmid; this is found, for example, in Qiagen®'s QE80 series of His-tag expression vectors.

9. Slow down the train. For high levels of protein expression, the rate of transcription needs to be coupled to that of translation, which in turn needs to be coupled to any essential co- or post-translational events, such as folding, cofactor binding or membrane insertion. When transcription outstrips translation, loss of cell viability can occur (along with the destruction of ribosomal RNA and induction of proteases; *see* Dong, *et al.*, 1995). There are several methods to tweak the rates of cellular metabolism to try to bring transcription, translation and post-translational processing in line. One can change promoters (Makrides, 1996; Baneyx, 1999). Strong promoters, such as T7, can be replaced with weaker promoters (arabinose, T5, tac). One can change cell lines. Two *E. coli* strains particularly suited for expression optimization, C41(DE3) and C43(DE3), were originally obtained from a selection for mutations that overcame lethality associated with over-expression from a T7 promoter (Miroux and Walker, 1996). These two strains also show an increase in plasmid stability relative to their parent BL21(DE3) strain (Dumon-Seignovert *et al.*, 2004). And, perhaps most easily, one can change the temperature during expression. For DsbA, the interplay between growth temperature and *E.coli* strain can be seen in Figure 1. Using BL21(DE3), slowing cell processes by growing at 25°C gives a large increase in expression over growth at 37°C (Lanes 9-10 vs. Lanes 7-8). In contrast, the strain C43(DE3), which is thought to have a mutation slowing the rate of transcription, gives a greater yield of expressed protein at 37°C (compare lanes 3-4 vs. 5-6). For DsbA C33S, we can choose between expressing at 37°C in C43, or expressing at 25°C in BL21(DE3). For some other proteins we have seen both C43(DE3) and a lower temperature are required for the highest expression level.

8. Eat yer spinach. If Popeye were a microbiologist, he might well claim that his cultures were "strong to the finish" because he feeds them iron. Studier recommends that trace metals be added to defined media, and, remarkably, that if a trace metal mixture is not available, 100 μM $FeCl_3$ alone will give the nearly the same level of expression (Studier, 2005). It is recommended that trace metals be added to rich media, as a precaution against batch-to-batch variation of trace metal content. We have found that addition of trace metals improves the yield of expressed protein even in BioExpress® 1000-supplemented (10 mL/L) media.

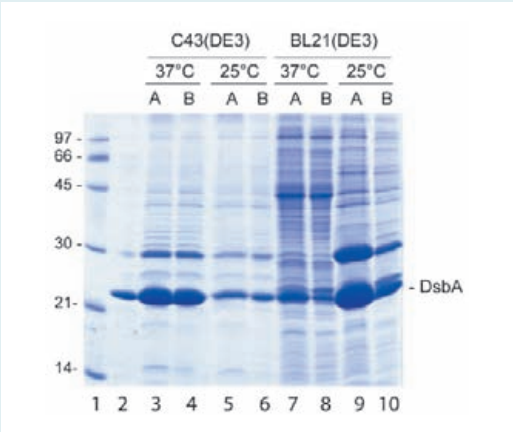


Figure 1. Expression of DsbA C33S in supplemented Studier Medium P containing BioExpress® 1000. DsbA C33S expression plasmid was transformed into chemically competent BL21(DE3)/pREP4 and C43(DE3)/pREP4. Several colonies were used to inoculate an overnight culture in LB containing 60 µg/mL ampicillin and 25 µg/mL kanamycin, which in turn was used to inoculate a pregrowth culture of LB. When the pregrowth culture reached an OD_{600} of 0.7, the cells were harvested in a sterile manner and resuspended in ¼ volume (relative to the pregrowth culture) of supplemented Medium P (Table 1). After 1 hour of growth, expression was induced with 0.8 mM IPTG, and growth continued at either 25 or 37°C, as indicated. The 37°C cultures were harvested at 4 hours, and the 25°C at 8 hours. The periplasmic proteins were obtained by resuspending the pellet from 100 mL of culture in 8 mL of 20% sucrose, 0.3 M Tris, pH 8, 0.5 mM EDTA, mixing at 4°C for 5 min, and repelleting the cells. Eight microliters of the supernatant from this centrifugation was loaded in the lanes labeled "A." The pellets were then resuspended in 4 mL water, mixed for 15 min, and centrifuged again. Four microliters of this supernatant was loaded in the lanes labeled "B." Lanes A and B, combined, for each *E.coli* strain (C43(DE3); BL21(DE3)) and induction temperature (25°C or 37°C) is representative of the total yield for each growth condition. Lane 1, Molecular mass standards (kDa); Lane 2, purified DsbA. Proteins were separated on a Tris-glycine (LaemmLi) gel containing 15% acrylamide and 2.5 M urea, and stained with Coomassie Blue.

7. Be media-savvy. Media acidification is detrimental to *E.coli* growth and protein yield (Swartz, 2001), but can be prevented to some extent by using a well-buffered medium with good aeration. We are currently using Studier Medium P (a phosphate-buffered medium) for expression of DsbA and several other proteins (Table 1). In addition, it has been found that calcium, although often added in relatively high amounts in traditional *E.coli* media as M9, is actually not essential at such high levels (and we have noted, can sometimes cause precipitant to form in the medium). On the other hand, Studier (2005) reports that using 2 mM $MgSO_4$, rather than 1 mM, can increase the cell density anywhere from 50% to five-fold – which would correspond to comparable increases in protein yield.

6. Exercise *aerobically*. The least expensive (and perhaps also least appreciated) supplement for cultures is oxygen, in the form of increased aeration. Glucose utilized by the aerobic respiratory chain provides more than 10 times the energy of glucose fermented. For growth on glycerol, the situation is even more dramatic: *E. coli* cannot utilize glycerol as an anaerobic energy source. And, as noted above, culture acidification, as occurs under anaerobic and microaerophilic conditions, is often detrimental to protein expression. To enhance aeration, we routinely use a culture volume of only 250 mL in a baffled 2 L flask. Note, however, that all expressions cannot tolerate all volume/baffling geometries, as certain expressions may cause cells to become fragile and sensitive to shear forces. Therefore optimal culture volume and rate of shaking with a given baffle geometry should be tested, not presumed.

5. Timing is everything. (part I: Induction) A common protocol for protein expression requires that IPTG be added when a culture has reached a cell density (A_{600}) of 0.8. But, following induction the growth of the culture will often slow or stop entirely. Thus it could happen that while the labeling medium supports uninduced growth to cell density of A_{600} = 4.0, the density of an induced culture ends up at harvest at only A_{600} = 1.0, giving perhaps only one quarter the potential yield. A better "rule of thumb" for time of induction would be to induce expression at 50% the density of an uninduced culture at harvest, and to also test the effect of induction at 80% uninduced density (i.e. at A_{600} = 3.2 for a culture that could grow to 4.0).

4. Timing is everything. (part II: Harvest) A typical protocol for a 37°C expression calls for harvest of *E. coli* cells 2-3 hours after protein expression is induced. However, it can be worthwhile to determine where expression is maximal, especially if one is using temperatures lower than 37°C. We now harvest our 25°C DsbA expression at 20 hours post-induction, and this more than doubles the protein yield of a six-hour harvest.

3. Quadruple up on the cells. Marley *et al.* (2001) have reported increases in expression of labeled protein by growing an *E. coli* culture in LB medium to density of A_{600} = 0.8, followed by harvest and resuspension at four-fold the density in labeling medium. Because cells containing the DsbA C33S expression plasmid grew well in rich medium, but poorly in labeling medium, this was an ideal solution for us. The four expression conditions of Figure 1 all used this method.

Table 1. Supplemented Studier Medium P, with BioExpress® 1000 adapted from Studier (2005)

Medium P salts pH ~7	nitrogen and carbon sources	cofactor and antibiotic supplements	"0.2x" Studier trace metals	
50 mM Na_2HPO_4	3 g/L (56 mM) NH_4Cl[1]	10 µg/mL thiamin	10 µM $FeCl_3$	0.4 µM $CuCl_2$
50 mM KH_2PO_4	4 g/L glucose[2]	10 µg/mL biotin	4 µM $CaCl_2$	0.4 µM $NiCl_2$
5 mM Na_2SO_4	10 mL/L BioExpress® 1000[3]	60 µg/mL ampicillin	2 µM $MnCl_2$	0.4 µM Na_2MoO_4
2 mM $MgSO_4$			2 µM $ZnSO_4$	0.4 µM Na_2SeO_3
			0.4 µM $CoCl_2$	0.4 µM H_3BO_3

1. For ^{15}N labeling: CIL # NLM-467, Ammonium chloride (^{15}N, 99%)
2. When following the method of Marley (2001) 4 g/L glucose is used, and otherwise 2 g/L glucose. For ^{13}C labeling: CIL # CLM-1396, D-glucose, (U-$^{13}C_6$, 99%)
3. For expression tests (unlabeled): CIL # CGM-1000-U-S, BioExpress® 1000 (unlabeled) 10x concentrate. For uniform ^{13}C,^{15}N labeling: CIL # CGM-1000-CN-S, BioExpress® 1000 (U-^{13}C, 98%; U-^{15}N, 98%), 10x concentrate.

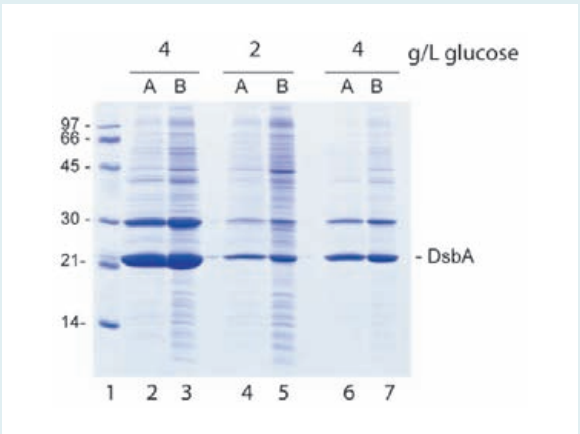


Figure 2. Comparison of expression of DsbA C33S in supplemented Studier Medium P containing BioExpress® 1000 using two concentrations of glucose. DsbA C33S was expressed as described in the legend to Figure 1, using *E.coli* strain BL21(DE3), at 25°C, with a harvest time of 14.5 hours post-induction. Lanes A and B are two fractions of DsbA obtained from each culture condition, and combined represent the total yield. Lanes 2, 3, 6, and 7 are from medium containing 4 g/L glucose; lanes 4 and 5 from 2 g/L glucose. Lanes 6 and 7 are a 1:5 dilution of the samples of lanes 2 and 3, respectively, demonstrating that the 4 g/L glucose culture contains at least 5x the DsbA of the culture with 2 g/L glucose. Lane 1 contains molecular mass standards (kDa). Proteins were separated on a Tris-glycine (Laemmli) gel containing 15% acrylamide and 2.5 M urea, and stained with Coomassie Blue.

2. Just a spoonful of sugar (or four). Marley *et al*. (2001) also reported using 4 g/L glucose in their dense cell cultures. We tested 2 g/L glucose and 4 g/L glucose and found an increase of more than five-fold in yield of DsbA C33S with a two-fold increase in glucose (Fig. 2). Additional glucose (6 and 8g/L) did further increase the yield, but not cost-effectively. At 4 g/L glucose, increasing the NH_4Cl from 2 g/L to 3 g/L gave a corresponding ~50% increase in expressed protein, but increase of NH_4Cl above 3 g/L had no further effect (data not shown).

1. Save both the baby *and* the bathwater. A common protocol for extracting periplasmic proteins such as DsbA directs that the cells be suspended in a hyperosmotic medium (we use 20% sucrose), followed by resuspension in water to burst the outer membrane, releasing the periplasmic protein. We found our DsbA overexpressing cells to be so fragile that breakage occured even in the sucrose solution (Fig. 1, lanes A) and had we not saved this supernatant, we would have lost a substantial part of our yield (compare Fig. 1, lanes B). Another occasion where it can be useful to save and assay all fractions is when assessing growth conditions and expression strains. A low-speed centrifugation of a well-sonicated *E.coli* sample will pellet inclusion bodies. If one is attempting to optimize for a high yield of protein in a native conformation, a large amount of protein in an inclusion body fraction can indicate that expression needs to be slowed somehow (see Tip #9 above) to allow time for proper folding or post-translational events.

By applying these tips to our DsbA C33S expression, we were able to produce sufficient ^{13}C,^{15}N labeled protein for solid-state NMR studies. Figure 3 shows a highly resolved 100 ms DARR spectrum of 5 mg microcrystalline DsbA taken on a 750 MHz spectrometer (Franks, *et al*. (2005)). We have found these tips to be generally useful – we have not only improved the expression of the *E. coli* periplasmic protein DsbA, but have been able to increase the expression yield of membrane proteins (e.g. *E. coli* cytochrome bo_3 oxidase (Frericks, *et al*. (2006) and DsbB (Li, *et al*. (2007)), as well as heterologous (non- *E. coli*) proteins expressed in *E. coli*.

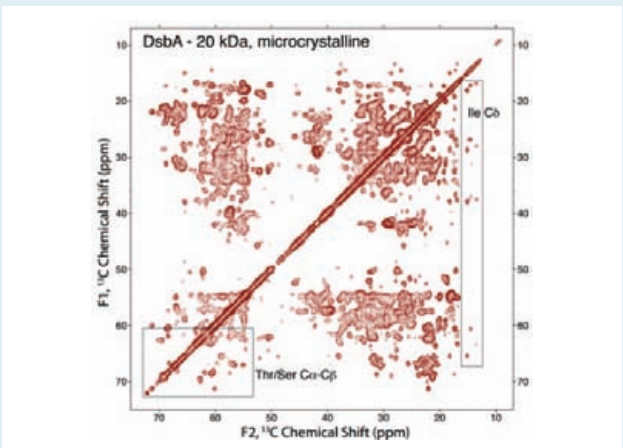


Figure 3. Solid-state NMR C-C 2D spectrum of microcrystalline DsbA acquired at 750 MHz with 100 ms DARR mixing. The aliphatic region of the spectrum is shown, with the particularly well-resolved isoleucine delta carbon correlations and serine and threonine Cα-Cβ correlations outlined in boxes. The sample is U-^{13}C,^{15}N- labeled DsbA (5 mg) in a 3.2 mm standard Varian rotor. Data was acquired for 18 hours at -10°C, utilizing 12.5 kHz magic-angle spinning and high power proton decoupling.

References

Baneyx, F. Recombinant protein expression in *Escherichia coli.* **1999**. *Curr Opin Biotech, 10,* 411-421.

Dong, H.; Nilsson, L.; and Kurland, C.G. Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. **1995**. *J Bacteriol*, *177,* 1497-1504.

Dumon-Seignovert, L.; Cariot, G.; and Vuillard, L. The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3). **2004**. *Protein Expr Purif*, *37,* 203-206.

Frericks, H.L.; Zhou, D.H.; Yap, L.L.; Gennis, R.B.; and Rienstra, C.M. Magic-angle spinning solid-state NMR of a 144 kDa membrane protein complex: *E. coli* cytochrome bo3 oxidase. **2006**. *J Biomol NMR, 36,* 55-71.

Franks, W.T.; Zhou, D.H.; Wylie, B.J.; Money, B.G.; Graesser, D.T.; Frericks, H.L.; Sahota, G.; and Rienstra, C.M. Magic-angle spinning solid-state NMR spectroscopy of the beta-1 immunoglobulin binding domain of protein G (GB1): 15N and 13C chemical shift assignments and conformational analysis. **2005**. *J Am Chem Soc, 127*, 12291-12305.

Kishigami, S.; Kanaya, E.; Kikuchi, M.; and Ito, K. DsbA-DsbB Interaction through their active site cysteines. Evidence from an odd cysteine mutant of DsbA. **1995**. *J Biol Chem*, *270,* 17072-17074.

Li, Y.; Berthold, D.A.; Frericks, H.L.; Gennis, R.B.; and Rienstra, C. Partial 13C and 15N chemical-shift assignments of the disulfide-bond-forming enzyme DsbB by 3D magic-angle spinning NMR spectroscopy. **2007**. *Chembiochem, 8*, 434-442.

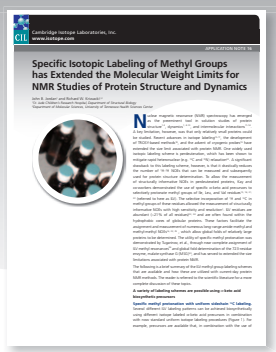
Makrides, S.C. Strategies for achieving high-level expression of genes in *Escherichia coli.* **1996**. *Microbiol Rev*, *60,* 512-538.

Marley, J.; Lu, M.; and Bracken, C. A method for efficient isotopic labeling of recombinant proteins. **2001.** *J Biomol NMR*, *20*, 71-75.

Miroux, B.; and Walker, J.E. Over-production of proteins in *Escherichia coli:* Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. **1996**. *J Mol Biol*, *260,* 289-298.

Studier, F.W. Protein production by auto-induction in high-density shaking cultures. **2005**. *Protein Expr Purif*, *41,* 207-234.

Swartz, J.R. Advances in *Escherichia coli* production of therapeutic proteins, **2001**. *Curr Opin Biotech*, *12,* 195-201.



Specific Isotope Labeling of Methyl Groups Has Extended the Molecular Weight Limits for NMR Studies of Protein Structure and Dynamics

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Nuclear magnetic resonance (NMR) spectroscopy has emerged as the preeminent tool in solution studies of protein structure,[1-3] dynamics,[1, 4-10] and intermolecular interactions.[11-14] A key limitation, however, was that only relatively small proteins could be studied. Recent advances in isotope labeling,[15-17] the development of TROSY-based methods,[18] and the advent of cryogenic probes[19] have extended the size limit associated with protein NMR. One widely used isotopic labeling scheme is perdeuteration, which has been shown to mitigate rapid heteronuclear (*e.g.* ^{13}C and ^{15}N) relaxation.[20] A significant drawback to this labeling scheme, however, is that it drastically reduces the number of ^{1}H-^{1}H NOEs that can be measured and subsequently used for protein structure determination. To allow the measurement of structurally informative NOEs in perdeuterated proteins, Kay and coworkers demonstrated the use of specific α-keto acid precursors to selectively protonate methyl groups of Ile, Leu, and Val residues[15,16,21,22] (referred to here as ILV). The selective incorporation of ^{1}H and ^{13}C in methyl groups of these residues allowed the measurement of structurally informative NOEs with high sensitivity and resolution[1]. ILV residues are abundant (~21% of all residues)[23, 24] and are often found within the hydrophobic cores of globular proteins. These factors facilitate the assignment and measurement of numerous long-range amide-methyl and methyl-methyl NOEs,[16, 22, 25] which allow global folds of relatively large proteins to be determined. The utility of specific methyl protonation was demonstrated by Tugarinov, *et al.*, through near complete assignment of ILV methyl resonances[26] and global fold determination of the 723-residue enzyme, malate synthase G (MSG),[23] and has served to extended the size limitations associated with protein NMR.

The following is a brief summary of the ILV methyl group labeling schemes that are available and how these are utilized with current-day protein NMR methods. The reader is referred to the scientific literature for a more complete discussion of these topics.

A variety of labeling schemes are possible using α-keto acid biosynthetic precursors

Specific methyl protonation with uniform sidechain ^{13}C labeling. Several different ILV labeling patterns can be achieved biosynthetically using different isotope-labeled α-keto acid precursors in combination with now standard uniform isotope-labeling procedures (Figure 1). For example, precursors are available that, in combination with the use of ^{15}N ammonium chloride, U-^{13}C,^{2}H-labeled glucose and $^{2}H_2O$, allow ^{13}C and ^{2}H labeling at non-methyl ILV sidechain sites and ^{13}C and ^{1}H labeling at one or both of the methyl groups of Val and Leu residues (in the form of α-ketoisovaleric acid giving $^{13}CH_3/^{13}CH_3$ labeling patterns) and at the δ1 methyl group of Ile (using α-ketobutyric acid) (Figure 1A). This labeling scheme is achieved by adding the sodium salts of α-ketobutyric and α-ketoisovaleric acids (60 mg/L and 100 mg/L, respectively) to otherwise standard $^{2}H_2O$-based minimal media about 1 hour prior to induction of protein expression with IPTG.[15,22,27] These precursors may be used together or separately without scrambling between the Ile and the Val/Leu residues.

This labeling scheme results in a continuous network of ^{13}C-^{13}C bonds between the polypeptide backbone and $^{13}C,^{1}H_3$ methyl groups of ILV residues and is compatible with NMR experiments that transfer magnetization from the methyl groups to other sidechain ^{13}C sites, to sites in the backbone and back,[26] or from methyl groups to sidechain ^{13}C sites.[27, 28] This labeling pattern and these experiments are used to assign ILV methyl resonances to specific amino acids in the primary sequence when sequential backbone resonance assignments have previously been established. Protein samples labeled in this manner can also be used to record 3D and 4D ^{13}C and/or ^{15}N-edited NOESY data, from which amide-methyl and methyl-methyl distance restraints can be derived and structures determined.[23, 27]

The utility of selective methyl protonation has been demonstrated by Lewis Kay's group at the University of Toronto. Kay's group developed ^{1}H-detected NMR experiments for use in making methyl group assignments in proteins with methyl protonated ILV spin systems.[26] These ^{1}H methyl-detected "out-and-back" experiments include the HMCM(CG)CBCA, Ile, Leu-HMCM(CGCBCA)CO, and Val-HMCM(CBCA)CO[26] experiments. These experiments are highly sensitive, and were acquired using a room-temperature probe at 800 MHz in 59, 58, and 21 hours,[26] respectively, with a 0.9 mM protein sample. The use of these experiments enabled the ^{1}H methyl ($^{1}H_{me}$) and ^{13}C methyl ($^{13}C_{me}$) resonances of nearly 78% of the ILV methyl groups in a 82 kDa protein (MSG) to be assigned.[26] Subsequently, the assignment of 3D and 4D NOESY experiments yielded distance-restraint information that, when combined with other types of structural restraints, allowed determination of a well-defined global fold for this 723-residue protein.[23]

More recently, a 3D ^{13}C-detected CH_3-TOCSY experiment was developed which allows correlation of $^{1}H_{me}$ and $^{13}C_{me}$ chemical shifts with $^{13}C_{aliphatic}$ chemical shifts in ILV-protonated spin systems. (Figure 2).[27] Jordan, *et al.*, used this experiment to obtain ILV methyl group chemical shift assignments for a 14 kDa protein domain. These assignments were subsequently used to assign 3D ^{15}N- and ^{13}C-NOESY-HSQC spectra recorded using the same ILV-^{13}C,^{1}H-labeled protein sample. The ^{13}C-detected CH_3-TOCSY experiment required the use of a cryogenic probe that can directly detect both ^{1}H and ^{13}C NMR signals and was acquired in ~16 hours with a 1 mM protein sample. The use of ^{13}C detection enabled very high resolution data to be acquired in the directly detected carbon dimension, facilitating the sequence-specific assignment ILV ^{13}C,^{1}H methyl resonances.[27] The limited distance information obtained from 3D NOESY spectra for the ILV-^{13}C,^{1}H labeled sample was sufficient to obtain a well-defined global fold of a 14 kDa protein.[27] In addition, this group also demonstrated that the CH_3-TOCSY experiment used with ILV-^{13}C,^{1}H labeling was effective in obtaining similar assignments for a 10 kDa protein in the context of a 42 kDa binary protein complex.

Selective ^{13}C,^{1}H labeling of ILV methyl groups to create isolated methyl spin probes. The α-ketobutyrate and α-ketoisovalerate methyl group precursors are also available with ^{13}C and ^{1}H incorporated only in the methyl groups (in either one or both of these in α-ketoisovalerate), with the remainder of the carbons present as ^{12}C with ^{2}H labeling (Figure 1B). This approach gives rise to isolated ^{13}C,^{1}H-labeled methyl groups for ILV residues without scalar coupling to other ^{13}C nuclei and, therefore, that exhibit singlet lineshapes (when using ^{1}H decoupling) with favorable relaxation properties. For L and V residues, methyl group relaxation properties are optimal when only one of the two methyl moieties is incorporated as ^{13}C,^{1}H. [26] While this reduces by half the amount of ^{13}C and ^{1}H isotope labels incorporated into each of the two methyl groups, signal loss is compensated for by improved relaxation, resulting in narrower methyl resonances.[26] Further, the elimination of methyl-methyl relaxation between the geminal methyl groups of L and V residues allows NOE interactions between these and other ILV methyl groups to be observed over long distances (e.g. up to 8 Å).[23] This labeling strategy is often combined with uniform ^{15}N and ^{2}H labeling of aliphatic sites other than ILV methyl groups labeling and allows long-range amide-methyl and methyl-methyl NOEs to be observed using appropriate 3D and 4D ^{13}C- and/or ^{15}N-edited NOESY NMR experiments.[23] The observation of long-range NOEs is optimized when all aliphatic sites other than ILV methyl groups are ^{12}C and ^{2}H labeled, which is achieved through biosynthetic labeling with U-^{12}C,^{2}H-glucose as the sole carbon source and $^{2}H_2O$ as the solvent.[29] If natural abundance glucose is used with $^{2}H_2O$ as the solvent, which is more economical, aliphatic sites (other than the specifically ^{1}H,^{13}C-labeled ILV methyl groups) will be ^{2}H-labeled to the extent of ~70%.

In addition to the applications described above (*e.g.* for optimal detection of long-range NOEs involving ILV methyl groups), specifically ^{1}H,^{13}C labeled ILV methyl groups have been used as reporters of protein-ligand interactions. This approach is an attractive complement to the use of ^{1}H,^{15}N amide groups as probes of interactions because 2D ^{1}H-^{13}C chemical shift correlation spectra can be recorded with high sensitivity and because small molecules, as well as macromolecules, often bind within hydrophobic pockets or on hydrophobic surfaces comprised of methyl groups. For proteins of known structure, this allows the binding sites for small molecules to be identified on the basis of binding-induced methyl group chemical shift perturbations. For small, ^{1}H,^{13}C ILV methyl-labeled proteins, 2D ^{1}H-^{13}C HSQC spectra can be recorded quickly (~10 minutes) with relatively dilute protein solutions (~50 μM). The use of cryogenic probes reduces these requirements further. Further, Kay and coworkers have shown that, for large proteins or protein assemblies (82 kDa and 305 kDa, respectively), 2D ^{1}H,^{13}C HMQC spectra exhibit superior sensitivity and resolution due to the "methyl TROSY" effect.[29, 30] For example, Hamel, *et al.*, described the use of methyl-TROSY NMR to map the residues at the protein-protein interface of the 120 kDa CheA-CheW complex[31], while Hajduk, *et al.*, demonstrated that the use of selective ILV methyl labeling in high-thoughput, small-molecule screening resulted in ^{1}H-^{13}C HSQC spectra with 3-5 times higher signal-to-noise ratios than the corresponding ^{1}H-^{15}N HSQC spectra.[32]

Selective ^{2}H,^{13}C labeling of ILV methyl groups for ^{2}H and ^{13}C NMR relaxation studies of protein dynamics. The topics discussed above are generally focused on using specifically labeled methyl groups to probe protein structure and to monitor protein-protein and protein-ligand interactions. It is well appreciated that methyl groups also serve as probes of side-chain dynamics within proteins.[33] In particular, studies of the relaxation behavior of ^{2}H[34, 35] and ^{13}C spins[36, 37] in uniformly ^{13}C and fractionally ^{2}H-labeled methyl groups have expanded our knowledge of protein side-chain dynamics. In these studies, the relaxation behavior of only one type of methyl isotopomer is monitored at a time. Because deuterons and protons are randomly incorporated into aliphatic sites during bacterial biosynthesis in the presence of $^{1}H_2O$ and $^{2}H_2O$, the concentration of molecules with a particular isotopomer at a particular site (*e.g.* ILV methyl groups) is only a fraction of the total protein concentration. Consequently, the incorporation of ILV methyl group isotopomers with uniform isotopic composition (for example $^{13}CHD_2$ (note: D ≡ ^{2}H here for clarity) for ^{2}H and ^{13}C relaxation studies) would improve spectral S/N and extend the application of these experiments to a broader range of protein systems (Figure 1C). In fact, Kay and coworkers have used this approach in their studies of MSG, an 82 kDa and 723-residue protein,[6, 26] as well as the 20 S proteasome with an aggregate mass of 670 kDa.[38]

Summary and outlook – new horizons await

A discussed at the outset, NMR spectroscopy is a powerful tool in studies of protein structure, protein dynamics, and the interaction of proteins with their ligands. The use of specific ILV methyl labeling was essential in extending the size limit for NMR-based

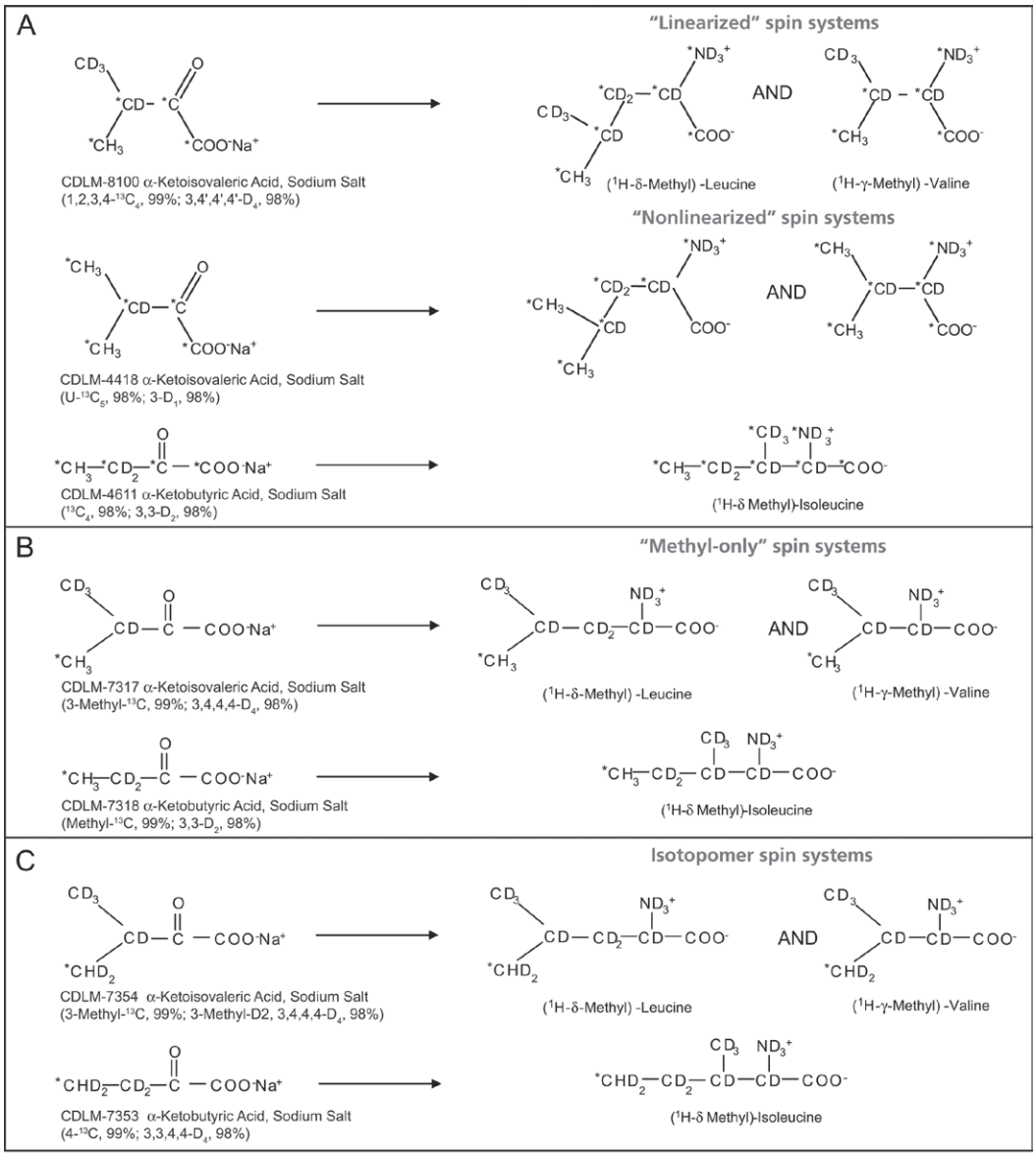
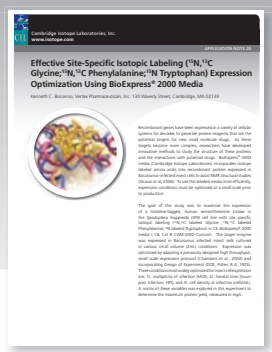


Figure 1. Differently isotope labeled α-ketoacid metabolic precursors of Ile, Leu and Val sidechains allow a variety of labeling patterns. Precursors used to produce ILV methyl-labeled proteins for (A) NMR experiments used to assign backbone and side chain resonances and for measuring long-range NOEs, (B) for NMR experiments used to measure long-range NOEs and for mapping ligand binding, and (C) for NMR relaxation experiments to study ILV methyl group dynamics.

global fold determination to ~80 kDa. By being both economically feasible and widely available, this isotope labeling strategy enables such challenging protein structural studies. As this labeling strategy is more widely adopted by the NMR/structural biology community, we look forward to future applications involving even more challenging biomolecular systems. As discussed above, applications extend beyond structure determination and ligand mapping. For example, in 2005, Kay and coworkers reported the use of Ile methyl labeling of the 300 kDa protease, ClpP, and methyl TROSY methods to detect a conformational exchange process on the millisecond timescale.[39] It was argued that this exchange process is involved in the opening of pores within the midline of the ClpP barrel-like structure for product release. This example illustrates how information from X-ray crystallography, which provided the overall structure of ClpP, and NMR spectroscopy, providing insights into dynamics directly related to key steps of the catalytic cycle, can be combined to reveal how complex molecular machines perform their biological functions. With these advancements in isotopic labeling and the implementation of new NMR methods enabled by them, the tools are in hand to extend our knowledge of the relationships between biomolecular structure, dynamics and function.

References

1. Kay, L.E. **2005**. *J Magn Reson, 173*, (2), 193-207.
2. Wuthrich, K. *NMR of Proteins and Nucleic Acids*. John Wiley & Sons: New York, **1986**.
3. Clore, G.M.; Gronenborn, A.M. **1991**. *Progress in NMR Spectroscopy, 23*, 43-92.
4. Palmer, A.G., **2001**. *3rd Annu Rev Biophys Biomol Struct, 30*, 129-55.
5. Kay, L.E. **1998**. *Nat Struct Biol, 5*, 513-516.
6. Tugarinov, V.; Kanelis, V.; Kay, L.E. **2006**. *Nature Protocols, 1*, 749-754.
7. Kern, D.; Eisenmesser, E.Z.; Wolf-Watz, M. **2005**. *Methods Enzymol, 394*, 507-24.
8. Wand, A.J. **2001**. *Nat Struct Biol, 8*, (11), 926-31.
9. Dyson, H J.; Wright, P.E. **2002**. *Adv Protein Chem, 62*, 311-40.
10. Dyson, H.J.; Wright, P.E. **2005**. *Methods Enzymol, 394*, 299-321.
11. Lian, L.Y.; Barsukov, I.L.; Sutcliffe, M.J.; Sze, K.H.; Roberts, G.C. **1994**. *Methods Enzymol, 239*, 657-700.
12. Pellecchia, M.; Sem, D.S.; Wuthrich, K. **2002**. *Nat Rev Drug Discov, 1*, (3), 211-9.
13. Betz, M.; Saxena, K.; Schwalbe, H. **2006**. *Curr Opin Chem Biol, 10*, (3), 219-25.
14. Bonvin, A.M.; Boelens, R.; Kaptein, R. **2005**. *Curr Opin Chem Biol, 9*, (5), 501-8.
15. Gardner, K.G.; Kay, L.E. **1998**. *Ann Rev Biophys Biomol Struct, 27*, 357-406.
16. Rosen, M.K.; Gardner, K.H.; Willis, R.C.; Parris, W.E.; Pawson, T.; Kay, L.E. **1996**. *J Mol Biol, 263*, (5), 627-636.
17. Goto, N.K.; Gardner, K.H.; Mueller, G.A.; Willis, R.C.; Kay, L.E. **1999**. *J Biomol NMR, 13*, (4), 369-74.
18. Pervushin, K.; Riek, R.; Wider, G.; Wuthrich, K. **1997**. *Proc Natl Acad Sci, 94*, 12366-12371.
19. Kovacs, H.; Moskau, D.; Spraul, M. **2005**. *Prog NMR Spec, 46*, 131-155.
20. Venters, R.A.; Huang, C.C.; Farmer, B.T., 2nd; Trolard, R.; Spicer, L.D.; Fierke, C.A. **1995**. *J Biomol NMR, 5*, (4), 339-344.
21. Kay, L.E.; Gardner, K.H. **1997**. *Curr Op Str Biol, 7*, 722-731.
22. Goto, N.K.; Kay, L.E. **2000**. *Curr Opin Struct Biol, 10*, (5), 585-592.
23. Tugarinov, V.; Choy, W.Y.; Orekhov, V.Y.; Kay, L.E. **2005**. *Proc Natl Acad Sci USA, 102*, (3), 622-627.
24. Tugarinov, V.; Kay, L.E. **2005**. *Chembiochem, 6*, (9), 1567-1577.
25. Metzler, W.J.; Leiting, B.; Pryor, K.; Mueller, L.; Farmer, B.T., 2nd. **1996**. *Biochemistry, 35*, (20), 6201-6211.
26. Tugarinov, V.; Kay, L.E. **2003**. *J Am Chem Soc, 125*, (45), 13868-13878.
27. Jordan, J.B.; Kovacs, H.; Wang, Y.; Mobli, M.; Luo, R.; Anklin, C.; Hoch, J.C.; Kriwacki, R.W. **2006**. *J Am Chem Soc, 129*, (28), 9119-9128.
28. Hu, K.; Vogeli, B.; Pervushin, K. **2005**. *J Magn Reson, 174*, (2), 200-208.
29. Tugarinov, V.; Kay, L.E. **2004**. *J Biomol NMR, 28*, (2), 165-72.
30. Tugarinov, V.; Hwang, P.M.; Ollerenshaw, J.E.; Kay, L.E. **2003**. *J Am Chem Soc, 125*, (34), 10420-8.
31. Hamel, D.J.; Dahlquist, F.W. **2005**. *J Am Chem Soc, 127*, (27), 9676-7.
32. Hajduk, P.J.; Augeri, D.J.; Mack, J.; Mendoza, R.; Yang, J.G.; Betz, S.F.; Fesik, S.W. **2000**. *J Am Chem Soc, 122*, 7898-7904.
33. Ollerenshaw, J.E.; Tugarinov, V.; Skrynnikov, N.R.; Kay, L.E. **2005**. *J Biomol NMR, 33*, (1), 25-41.
34. Millet, O.; Muhandiram, D.R.; Skrynnikov, N.R.; Kay, L.E. **2002**. *J Am Chem Soc, 124*, (22), 6439-48.
35. Mulder, F.A.; Hon, B.; Mittermaier, A.; Dahlquist, F.W.; Kay, L.E. **2002**. *J Am Chem Soc, 124*, (7), 1443-51.
36. Teilum, K.; Brath, U.; Lundstrom, P.; Akke, M. **2006**. *J Am Chem Soc, 128*, (8), 2506-7.
37. Lundstrom, P.; Akke, M. **2005**. *Chembiochem, 6*, (9), 1685-92.
38. Sprangers, R.; Kay, L.E. **2007**. *Nature, 445*, (7128), 618-22.
39. Sprangers, R.; Gribun, A.; Hwang, P.M.; Houry, W.A.; Kay, L.E. **2005**. *Proc Natl Acad Sci USA, 102*, (46), 16678-83.



Effective Site-Specific Isotopic Labeling (^{13}C,^{15}N Glycine; ^{13}C,^{15}N Phenylalanine; ^{15}N Tryptophan) Expression Optimization Using BioExpress® 2000 Media

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Recombinant genes have been expressed in a variety of cellular systems for decades to generate protein reagents that are the potential targets for new small molecule drugs. As these targets become more complex, researchers have developed innovative methods to study the structure of these proteins and the interactions with potential drugs. BioExpress® 2000 media (Cambridge Isotope Laboratories) incorporates isotope-labeled amino acids into recombinant protein expressed in Baculovirus-infected insect cells to assist NMR structural studies (Strauss *et al.,* 2006). To use this labeled media most efficiently, expression conditions must be optimized at a small scale prior to production.

The goal of this study was to maximize the expression of a histidine-tagged, human serine/threonine kinase in the *Spodoptera frugiperda* (SF9) cell line with site-specific isotopic labeling (^{15}N,^{13}C-labeled Glycine ;^{15}N,^{13}C-labeled Phenylalanine; ^{15}N-labeled Tryptophan) in CIL BioExpress® 2000 media (CIL Catalog # CGM-2000-Custom). The target enzyme was expressed in Baculovirus-infected insect cells cultured in various small volume (2 mL) conditions. Expression was optimized by adapting a previously designed high throughput, small scale expression protocol (Chambers *et al*., 2004) and incorporating Design of Experiment (DOE, Fisher, R.A.,1925). Three conditions most widely optimized for insect cell expression are: 1). multiplicity of infection (MOI), 2). harvest time [hours post infection, HPI], and 3). cell density at infection (cells/mL). A matrix of these variables was explored in this experiment to determine the maximum protein yield, measured in mg/L.

A Box-Behnken response surface model (Box & Behnken, 1960) was applied in the experimental design using three equally spaced factors (Low, Medium, High) for each of the three conditions (MOI, HPI, Cell Density) in order to study the quantitative response, protein yield. This model provides 13 conditions including a centerpoint with replicates. DOE allows the exploration of many variables via a limited number of experimental conditions using a statistically significant model produced by the experimental data. (Chambers & Swalley, 2008).

Cells were grown in 24 well block microplates (Whatman plc, UK) and infected with Baculovirus at cell density and MOI specified by the Box-Behnken design. Cell cultures were pelleted and frozen upon harvest. Cell lysates were solubilized and the target protein was purified using nickel magnetic agarose beads (Sigma-Aldrich, MO). Protein yield was determined using a dot blot assay with an anti-histidine primary antibody (BD BioSciences, NJ) and a Goat anti-Mouse secondary antibody that fluoresces at the 680 nm wavelength (LI-COR Biosciences, NE) using the Odyssey® Infrared Imaging System and the analysis software provided (LI-COR Biosciences, NE) (Figure 1).

The expression data was analyzed with statistical software (JMP™ v6.0, NC) to determine if the data sufficiently fit the model (Figure 2). The Box-Behnken model was verified since yield data provided adequate fit (R^2=80%) and there were significant regression factors in the model (p_{yield}<0.001). Additionally, the p-value for the lack of fit test is not significant (p>0.05). The data predicted optimal conditions for the large scale expression to generate a yield of 1.3 ± 0.1 mg/L, a 20% improvement from the best condition in the screen. As the case with many experiments incorporating DOE, the optimal condition from the model (MOI=5, HPI=48, Cell Density=1.5 x 10^6 cells/mL) corresponds to conditions not actually tested in the initial experiment. Before producing a large scale bioreactor production batch, a 0.5L scale expression experiment was carried out at the optimal conditions to validate the model. A 2.8 liter Fernbach flask was used for the validation experiment since it provides comparable results to a bioreactor. Standard media was used in lieu of BioExpress® 2000-labeled media for the validation since the small scale study indicated that expression levels were comparable between the two media types at 69% of the conditions as indicated by the Student's test one-way analysis of variance (ANOVA) (Figure 1). The best experimental condition and the optimal condition predicted by the model were expressed. The target protein was purified using nickel affinity resin (Sigma-Aldrich, MO) and protein yield quantified using LC90 Lab Chip technology (Caliper Inc., MA) to validate the yields obtained in the small scale screen. The model's predicted expression condition produced a 40% greater yield than the best experimental condition in the standard media (Figure 3).

The hypothesized optimal expression conditions (MOI=5, HPI=48, Cell Density=1.5 x 10^6 cells/mL) were used for production in the BioExpress® 2000 media using protocols recommended by CIL. One liter of cells was cultured in a 2 L Wave Bioreactor bag for each batch (GE Healthcare, NJ). The final yield of the affinity purified protein was 1.3 mg/L, within error of the model's prediction for labeled media. Figure 4 describes the process implemented from screening through validation into production.

It is important to connect laboratory technique and procedures with statistical methods to maximize the experimental efficiency at every level of experimentation. Incorporating isotopically labeled

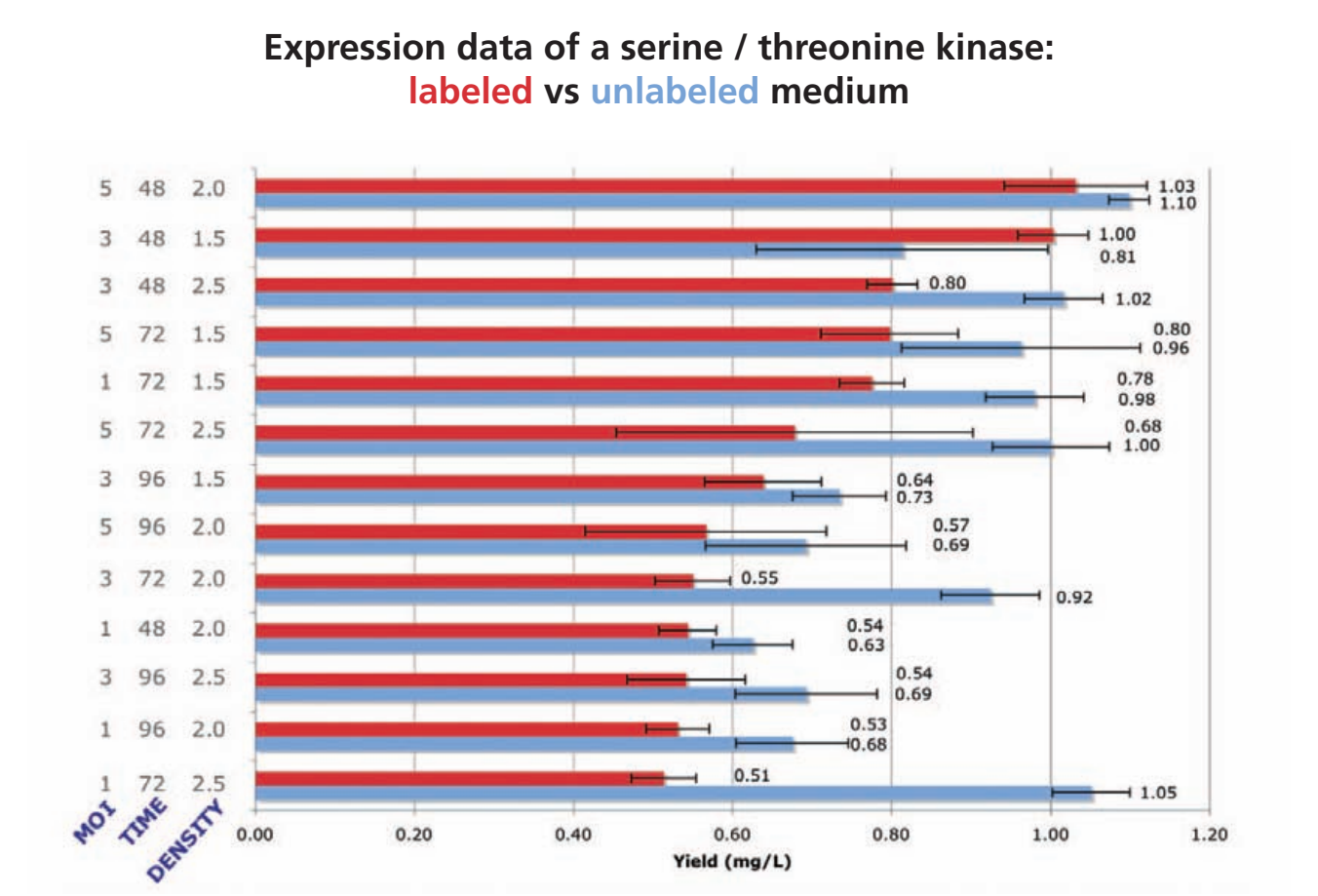

Figure 1. Site-specific isotopic labeling in BioExpress® 2000 media (red) and unlabeled expression in a standard media (blue, ESF921, Expression Systems, CA). Student's t- test indicates BioExpress® 2000 media provides comparable expression yields to those of a standard media at 69% of the conditions used in the DOE experiment. (Box, Hunter, Hunter, 1978).

amino acids into insect cell media increases the cost of the media; therefore, optimal expression conditions for the target protein are desired before advancing to this media. DOE and statistical analysis control the size of the experiment and allow efficient use of reagents, such as labeled media, for optimization purposes. This enables more of the labeled media to be used for the scale up processes and ultimately to supply material to generate NMR samples. Designing a screening experiment using high throughput miniaturization and sound statistical models minimizes reagent consumption while maximizing the expression yields of protein production batches.

Acknowledgements

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Optimization of labeled expression in insect cells using design of experiment (DOE)

Variables Explored	Low	Mid	High
Multiplicity of Infection (MOI)	1	3	5
Cell Density (10^6 cells/ml)	1.5	2.0	2.5
Time of Infection (hrs)	48	72	96

Green Represents Optimal Conditions

Response Surface Model (RSM) used to identify optimum conditions

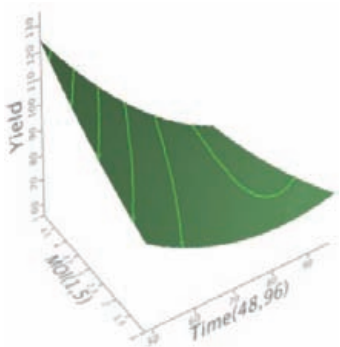

Figure 2. Insect cell expression of a recombinant human serine/threonine kinase with residue specific $^{15}N/^{13}C$ labeling in SF9 cells. **A.** A Box-Behnken design was used to maximize expression. **B.** The contour profile represents the Response Surface Model from the data, showing that the optimal predicted yield at cell density 1.5 x 10^6 cells/mL would be achieved at high MOI and low time.

Abbreviations

ANOVA – one-way analysis of variance
DOE – Design of experiment
HPI – hours post infection
MOI – multiplicity of infection
RSD – response surface design
RSM – response surface model
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF9 – *Spodoptera frugiperda*

Optimized Expression Condition Validation

	Predicted Condition from RSD	RSD Optimal Condition
Multiplicity of Infection (MOI)	5	5
Cell Density (10^6 cells/ml)	1.5	2.0
Time of Infection (hrs)	48	48
Small Scale Yield (mg/L)	Not Tested	1.1
Validation Yield (mg/L)	2.2	1.6

Figure 3. Caliper LC90 quantitation and virtual SDS-PAGE representation of the validation expression experiment. The DOE predicted condition produced a 40% greater yield than the best condition from the model and a 100% improvement to the RSD optimal yield from the small scale experiment.

Expression Strategy for Labeling in Insect Cells

Expression Scale	Volume (ml)	Vessel	Media	Yield (mg/L)	Analysis
DOE Screening	2	Deep Well Block	Cambridge Isotopes BioExpress 2000	0.5 - 1.0	DOT BLOT
Validation	500	Shake-Flask	Expression Systems ESF921	2.2	SDS-PAGE
Production	2000	Wave-Reactor	Cambridge Isotopes BioExpress 2000	1.3	SDS-PAGE

Figure 4. Expression summary. DOE screening occurred in a high throughput format and was analyzed using infrared fluorescence Dot Blot with Li-Cor quantitation. Validation and production yields were analyzed and quantified with Caliper LC90, virtual SDS-PAGE shown above. Validation confirmed the optimal condition defined by the DOE experiment, using unlabelled medium. Large-scale expression was then performed using the optimal condition defined by the DOE experiment using labeled medium.

Additional Products of Interest

Catalog No.	Description
CGM-2000-U	BioExpress® 2000 Unlabeled
CGM-2000-U-s	BioExpress® 2000 Unlabeled (200 mL media kit)
CGM-2000-N	BioExpress® 2000 (U-^{15}N, 98%)
CGM 2000-CN	BioExpress® 2000 (U-^{13}C, 98%; U-^{15}N, 98%)
DLM-2622	DL-1,4-dithiothreitol (DTT) (D_{10}, 98%)
DLM-2274	Dodecylphophocholine (DPC) (D_{38}, 98%)
DLM-197	Sodium dodecyl sulfate (SDS) (D_{29}, 98%)
DLM-1814	Tris(hydroxymethyl)methylamine (TRIS) (D_{11}, 98%)
DLM-3033	Imidazole (D_4, 98%)
DLM-3786	N-(2-Hydroxyethyl)Piperazine-N'-2-ethanesulfonic acid (HEPES) (D_{18}, 98%)
CNLM-2408-1	GFL Peptide Standard (^{13}C, 98%; ^{15}N, 96-99%)
CNLM-6840-10	SH3 Domain Protein (U-^{13}C, 98%; U-^{15}N, 98%)
NLM-7361-5	Ubiquitin (Algal) (U-^{15}N, 98%)
CNLM-7362-5	Ubiquitin (Algal) (U-^{13}C, 98%; U-^{15}N, 98%)

References

Box, George; and Behnken, Donald. Some new three-level designs for the study of quantitative variables. **1960**. *Technometrics, 2*, 455-475.

Chambers S.P.; Austen, D.A.; Fulghum, J.R.; and Kim, W.M. High-throughput screening for soluble recombinant expressed kinases in *Escherichia coli* and insect cells. **2004**. *Protein Expression and Purification, 36,* 40-47.

Strauss, André; Fendrich, Gabriele; and Jahnke, Wolfgang. Effective uniform isotope labeling of proteins expressed in Baculovirus-infected insect cells using BioExpress® 2000 (Insect cell) medium. **2006**. *Cambridge Isotope Laboratories Application Note 14,* 1-3.

Chambers S.P.; and Swalley, S. **2008**. Designing Experiments for High-Throughput Protein Expression. High-Throughput Protein Expression and Purification: Methods in Molecular Biology. Ed. *498,* 19-29.

Fisher, R.A. **1925**. Statistical Methods for Research Workers (ISBN 0-05-002170-2)

Box, George E.P.; Hunter, William G.; and Hunter, J. Stuart. **1978**. Statistics for Experimenters: An Introduction to Design, Data Analysis and Model Building (ISBN 0-471-09315-7)

[2,3-$^{13}C_2$]-Labeled Aromatic Residues as a Means to Improving Signal Intensities and Kick-Starting the Assignment of Membrane Proteins by Solid-State MAS-NMR

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Over the past few years solid-state MAS-NMR has rapidly been developing into a structure determination technique for biological macromolecules.[1-6]. Its advantages include the ability to study membrane proteins in their native lipid environment,[7-10] as well as making possible the study of non-soluble or non-crystallisable protein states, such as amyloid fibirils.[11-15] However, a prerequisite for structure determination is a high level of resonance assignment. There are numerous examples of small and medium-sized proteins for which this has been possible,[4,16-20] but for large membrane proteins, such as the 281-residue outer membrane protein G (OmpG)[21-22] resonance assignment still remains a challenge. Signal overlap is an obvious problem, but fast longitudinal and transverse relaxation rates also contribute towards lower signal/noise ratios. Furthermore, membrane proteins can reduce the Q-factor of the coil and the experimentalist is then left with the difficult task of balancing increased decoupling powers against possible sample heating which could lead to sample degradation. These problems result in lower signal intensities in proton-driven spin diffusion (PDSD) spectra, however, they can be addressed using several different strategies, such as by improving coil design,[23-25] by developing spectral editing pulse sequences,[26-27] or by using novel labeling strategies.[28-29] It is this last approach, using a novel labeling strategy, which is presented here in this application note.

It has been shown that samples produced using [1,3-$^{13}C_2$]- and [2-^{13}C]-labeled glycerol in the bacterial growth medium give rise to spectra with improved cross-relaxation properties and which prove very helpful at the restraint generation stage.[1] But assignment using solely these samples is not possible, since the C′, Cα and Cβ atoms are never simultaneously labeled. An alternative approach would be to use [1,2,3-$^{13}C_3$]-, [1,2-^{13}C]- and/or [2,3-$^{13}C_2$]-labeled amino-acids. This would retain the favorable cross-relaxation properties of the glycerol samples by keeping the number of consecutively bonded labeled carbon atoms low, but would provide a more useful set of labels for assignment. The two former labeling patterns can easily be generated with uniformly labeled short amino acids such as Alanine, Serine, Cysteine or Glycine. Additional incorporation of other amino acids with [2,3-$^{13}C_2$]-labeling would increase the number of detectable amino acids but reduce the overlap in NCO-type spectra,[30] thus providing scope for many unambiguous sequential assignments from these spectra.

When considering which amino acid types to select for [2,3-$^{13}C_2$]-labeling, it is worth noting that Cβ signals from aromatic amino acids are often very weak or not visible at all. [2,3-$^{13}C_2$]-labeling aromatic amino acids would reduce J-couplings at the Cα and Cβ positions, as well as ensuring that the aromatic ring (which undergoes fast relaxation) does not draw away magnetization and effectively act as a magnetization "sink." The [2,3-$^{13}C_2$]-labeling would then not only aid assignment, but also improve the spectral quality and identification of aromatic spin systems.

This application note describes the use of a novel labeling strategy to increase the spectral quality and level of resonance assignment in MAS-NMR spectra of the biological macromolecule, OmpG. Based on the protein's sequence, a labeling scheme of fully [^{15}N,^{13}C]-labeled Ala and Gly and [2,3-$^{13}C_2$,^{15}N]-labeled Tyr and Phe was selected. The sample (hereafter referred to as OmpG-GAFY) was prepared using established protocols and incorporating [2,3-$^{13}C_2$,^{15}N]-Tyr **(CIL Catalog # CNLM-7610)**, [2,3-$^{13}C_2$,^{15}N]-Phe **(CIL Catalog # CNLM-7611)**, [U-$^{13}C_3$,^{15}N]-Ala **(CIL Catalog # CNLM-534)** and [U-$^{13}C_2$,^{15}N]-Gly **(CIL Catalog # CNLM-1673)**. Initial spectra showed that the ^{13}C labels were incorporated as expected, with only minor contributions of labeled Serine produced *in vivo* from the labeled Glycine (Figure 1).

Improved signal intensities for [2,3-$^{13}C_2$]-labeled aromatic residues.

The gain in signal intensity for the Phe and Tyr residues was assessed by comparing spectra of OmpG-GAFY with those of uniformly labeled OmpG (OmpG-uni). Figure 2 shows that the number and intensity of aromatic Cα/Cβ peaks has increased for the OmpG-GAFY sample. The intensity increases could come from the removal of J-couplings in OmpG-GAFY as well as from the fact that the aromatic ring is no longer able to act as a 'magnetization sink'. The line widths were found to be similar for both samples which suggests that the latter process is the more dominant.

An alternative approach to improving the Cα/Cβ cross-peaks of aromatic residues is the use of band-selective experiments. A DREAM spectrum[31,32] of OmpG-uni has drastically improved signal intensities (by a factor of 10 or more) which are slightly higher than those in the OmpG-GAFY PDSD. A DREAM spectrum of OmpG-GAFY on the other hand only increases the signal

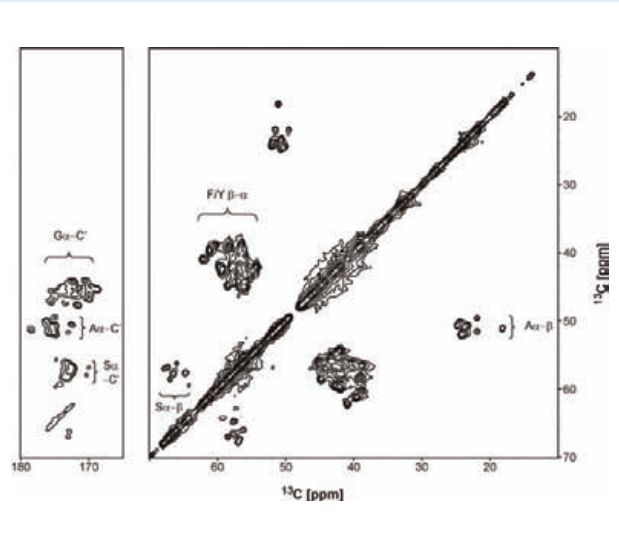

Figure 1. PDSD spectrum recorded on OmpG-GAFY with a 20 ms mixing time at 900 MHz. Residue and atom types are indicated for the each peak cluster.

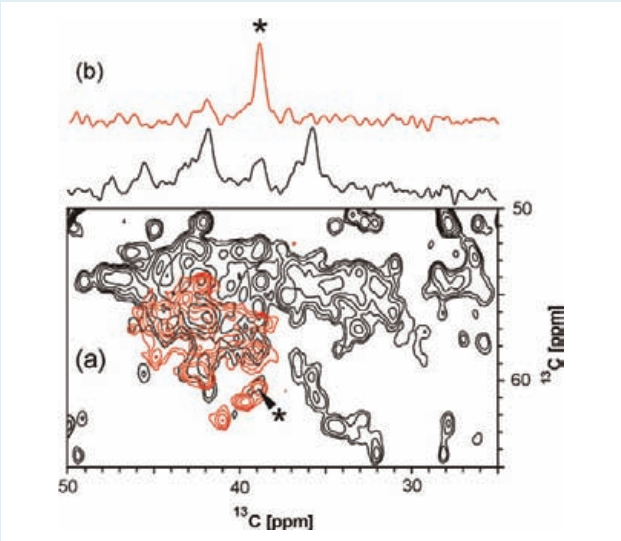

Figure 2. (a) Overlay of PDSD spectra recorded for OmpG-uni (black) and OmpG-GAFY (red) at 900MHz. (b) 1D traces of the spectra in (a) taken through the peak marked with a *.

intensity by a factor of 2. This supports the fact that the isolation of the Cα/Cβspin system from the side chain is the main source of signal enhancement when using [2,3-$^{13}C_2$]-labeled residues.

A major advantage of the OmpG-GAFY sample containing [2,3-$^{13}C_2$]-labeled aromatic residues is thus the fact that peak intensities nearly reach those of an OmpG-uni band-selective experiment, even during broadbanded experiments. This becomes particularly important when trying to obtain long-range as well as numerous sequential cross-peaks which require long mixing times. Using DREAM or other band-selective pulse sequences for this purpose would be inadvisable because the intense and continued irradiation over a long mixing time would lead to increased sample heating and thus risk sample degradation. In addition, band-selective experiments have the disadvantage that only those cross-peaks selected for are visible. Several spectra would have to be recorded to obtain the information content of a single broadbanded experiment. Samples containing [2,3-$^{13}C_2$]-labeled residues therefore offer an attractive way of improving signal intensities in broadbanded experiments which form an important part of the assignment and structure determination process of a protein.

Sequence-specific assignment

Initially spin systems were identified using a 3D NCACX spectrum recorded using a 20 ms PDSD mixing time. Some overlap could not be resolved, but all alanines, 96% of the glycines and 85% of the aromatics were identified. Inter-residue connections were then identified using a variety of 2D and 3D spectra: 3D NCACX spectra with long mixing times (300-500 ms) contained N_i-$C\alpha_i$-$C\alpha_j$ type peaks (where j is a neighboring residue or one close in space to i), PDSD spectra (400-700 ms) gave rise to Cα-Cα, Cα-Cβ and Cβ-Cβ peaks, and a 2D NCOCX spectrum (50 ms) and a REDOR spectrum (1 ms) provided unambiguous inter-residue correlations of the type N_i-C'_{i-1}, N_i-C'_{i-1}-$C\alpha_{i-1}$ and N_i-C'_{i-1}-$C\beta_{i-1}$. Starting points generally came from the PDSD and NCOCX/REDOR spectra. The NCACX spectra could then be used to identify further atoms in each spin system and link the other spectra. Figure 3 illustrates the assignment for the GGF motif. Further 2D PDSD-DARR, 3D NCACX and 3D NCOCX spectra were recorded for OmpG samples made using [1,3-$^{13}C_2$] and [2-^{13}C]-labeled glycerol.[1] The [2-^{13}C]-glycerol NCACX spectrum was used to distinguish between Phe and Tyr residues on the basis of N-Cα-Cγ peaks. Overall, these spectra were used to corroborate and extend the assignments obtained from the OmpG-GAFY sample leading to the identification of 45 sequence-specific assignments.

Conclusions

The GAFY labeling scheme which incorporates small uniformly labeled amino acids and [2,3-$^{13}C_2$]-labeled aromatic residues has provided a highly successful starting point for the sequence-specific assignment of the large membrane protein OmpG. The main advantage of the labeling scheme is its composition of a low number of small, isolated spin systems (pairs and triplets of sequentially labeled carbon atoms). This limits transfer of magnetization into the side chain resulting in enhanced spectral quality. In addition, the number of inter-residue cross-peaks is significantly increased which is important both for the assignment and structure calculation stages. The use of a mixture of [2,3-$^{13}C_2$]- and short [U-^{13}C]-labeled amino acids has eased the interpretation

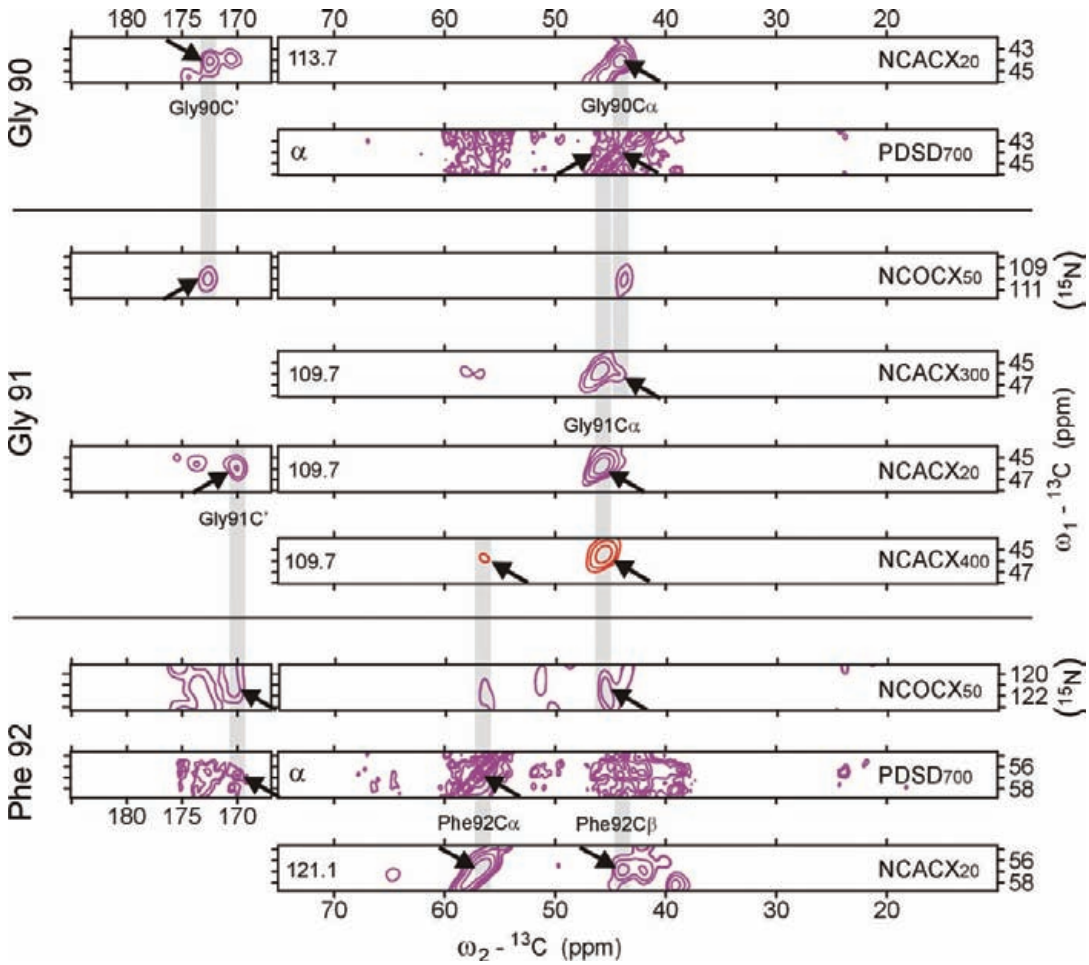

Figure 3. Spectra illustrating the assignment of the G90-G91-F92 motif in OmpG: Strips from 3D NCACX, 2D NCOCX and 2D PDSD spectra are shown with the spectrum type and mixing time (in ms) indicated on the right. The ppm value of the 3D NCACX ^{15}N planes and carbon type of the PDSD strips is indicated on the left. Purple spectra were recorded on OmpG-GAFY, red spectra on OmpG produced from [2-^{13}C]-glycerol. Cross-peaks which define each residue and link neighboring ones are indicated by arrows and highlighted in grey. The carbonyl regions of the 50 ms NCOCX spectrum are replaced with a better quality 1 ms REDOR spectrum.

of NCO- and NCA-type of spectra. In addition, this labeling scheme has the advantage compared to the [1,3-$^{13}C_2$]- and [2-^{13}C]-glycerol ones, that there is a direct connection between the Cα and Cβ chemical shifts by which the spin systems can be identified. Furthermore, [2,3-$^{13}C_2$]-labeling provides the spectral quality of band-selective experiments while allowing broadband experiments to be recorded, thus improving and increasing the information content of spectra.

Acknowledgments

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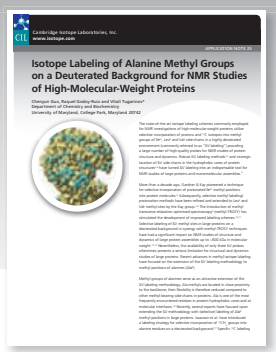
Additional products of interest:

CLM-1397	2-^{13}C Glycerol
CLM-1857-K	1,3-$^{13}C_2$ Glycerol

References

1. Castellani, F.; van Rossum, B.; Diehl, A.; Schubert, M.; Rehbein, K.; Oschkinat, H. **2002**. *Nature, 420*, 98-102.
2. Seidel, K.; Etzkorn, M.; Heise, H.; Becker, S.; Baldus, M. **2005**. *Chem Biochem, 6*, 1638-1647.
3. Zech, S. G.; Wand, A. J.; McDermott, A. E. **2005**. *J Am Chem Soc, 127*, 8618-8626.
4. Lange, A.; Becker, S.; Giller, K.; Pongs, O.; Baldus, M. **2005**. *Angew Chem Int Ed Engl, 44*, 2089-2092.
5. Loquet, A.; Bardiaux, B.; Gardiennet, C.; Blanchet, C.; Baldus, M.; Nilges, M.; Malliavin, T.; Böckmann, A. **2008**. *J Am Chem Soc, 130*, 3579-3589.
6. Franks, W.T.; Wylie, B.J.; Frericks Schmidt, H.L.; Nieuwkoop, A.J.; Mayrhofer, R.M.; Shah, G.J.; Graesser, D.T.; Rienstra, C.M. **2008**. *Proc Nat Ac Sci, 105*, 4621-4626.
7. Hiller, M.; Krabben, L.; Vinothkumar, K.R.; Castellani, F.; van Rossum, B.J.; Kühlbrandt, W.; Oschkinat, H. **2005**. *Chem Biochem, 6*, 1679-1684.
8. Lorch, M.; Fahem, S.; Kaiser, C.; Weber, I.; Mason, A.J.; Bowie, J.U.; Glaubitz, C. **2005**. *Chem Biochem, 6*, 1693-1700.
9. Andronesi, O.C.; Becker, S.; Seidel, K; Heise, H.; Young, H.S.; Baldus, M. **2005**. *J Am Chem Soc, 127*, 12965-12974.
10. Schneider, R.; Ader, C.; Lange, A.; Giller, K.; Hornig, S.; Pongs, O.; Becker S.; Baldus,M. **2008**. *J Am Chem Soc, 130*, 7427-7435.
11. Petkova, A.T.; Ishii, Y.; Balbach, J.J.; Antzutkin, O.N.; Leapman, R.D.; Delaglio, F.; Tycko, R. **2002**. *Proc Nat Ac Sci, 99*, 16742-16747.
12. Jaroniec, C.P.; MacPhee, C.E.; Bajaj, V.S.; McMahon, M.T.; Dobson, C.M.; Griffin, R.G. **2004**. *Proc Nat Ac Sci, 101*, 711-716.
13. Heise, H.; Hoyer, W.; Becker, S.; Andronesi, O.C.; Riedel, D.; Baldus, M. **2005**. *Proc Nat Ac Sci, 102*, 15871-15876.
14. Ferguson, N.; Becker, J.; Tidow, H.; Tremmel, S.; Sharpe, T.D.; Krause, G.; Flinders, J.; Petrovich, M.; Berriman, J.; Oschkinat, H.; Fersht, A.R. **2006**. *Proc Nat Ac Sci, 103*, 16248-16253.
15. Wasmer, C.; Lange, A.; Van Melckebeke, H.; Siemer, A.B.; Riek, R.; Meier, B.H. **2008**. *Science, 319*, 1523-1526.
16. Pauli, J.; Baldus, M., van Rossum, B.; de Groot, H.; Oschkinat, H. **2001**. *Chem Biochem, 2*, 272-281.
17. Böckmann, A.; Lange, A.; Galinier, A.; Luca, S.; Giraud, N.; Juy, M.; Heise, H.; Montserret, R.; Penin, F.; Baldus, M.**2003**. *J Biomol NMR, 27*, 323-339.
18. Igumenova, T.I.; McDermott, A.E.; Zilm, K.W.; Martin, R.W.; Paulson, E.K.; Wand, A.J. **2004**. *J Am Chem Soc, 126*, 6720-6727.
19. Goldbourt, A.; Gross, B.J.; Day, L.A.; McDermott, A.E. **2007**. *J Am Chem Soc, 129*, 2338-2344.
20. Franks, W.T.; Zhou, D.H.; Wylie, B.J.; Money, B.G.; Graesser, D.T.; Frericks, H.L.; Sahota, G.; Rienstra, C.M. **2005**. *J Am Chem Soc, 127*, 12291-12305.
21. Fajardo, D. A.; Cheung, J.; Ito, C.; Sugawara, E.; Nikaido, H.; Misra, R. **1998**. *J Bacteriol, 180*, 4452-4459.
22. Misra, R.; Benson, S. A. **1989**. *J Bacteriol, 171*, 4105-4111.
23. Stringer, J.A.; Bronnimann, C.E.; Mullen, C.G.; Zhou, D.H.H.; Stellfox, S.A.; Li, Y.; Williams, E.H.; Rienstra, C.M. **2005**. *J Magn Reson, 173*, 40-48.
24. Doty, F.D.; Kulkarni, J.; Turner, C.; Entzminger, G.; Bielecki, A. **2006**. *J Magn Reson, 182*, 239-253.
25. Gor'kov, P.L.; Chekmenev, E.Y.; Li, C.G.; Cotten, M.; Buffy, J.J.; Traaseth, N.J.; Veglia, G.; Brey, W. W. **2007**. *J Magn Reson, 185*, 77-93.
26. Jehle, S.; Hiller, M.; Rehbein, K.; Diehl, A.; Oschkinat, H.; van Rossum, B.J. **2006**. *J Biomol NMR, 36*, 169-177.
27. Jehle, S.; Rehbein, K.; Diehl, A.; van Rossum, B.J. **2006**. *J Magn Reson, 183*, 324-328.
28. Hong, M.; Jakes, K. **1999**. *J Biomol NMR,* 14, 71-74.
29. Etzkorn, M.; Martell, S.; Andronesi, O.C.; Seidel, K.; Engelhard, M.; Baldus, M. **2007**. *Angew Chem Int Ed Engl, 46*, 459-462.
30. Baldus, M.; Petkova, A.T.; Herzfeld, J.; Griffin, R.G. **1998**. *Mol Phys, 95*, 1197-1207.
31. Verel, R.; Baldus, M.; Ernst, M.; Meier, B.H. **1998**. *Chem Phys Lett, 287*, 421-428.
32. Verel, R.; Ernst, M.; Meier, B.H. **2001**. *J Magn Reson, 150*, 81-9.



Isotope Labeling of Alanine Methyl Groups on a Deuterated Background for NMR Studies of High-Molecular-Weight Proteins

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The state-of-the-art isotope-labeling schemes commonly employed for NMR investigations of high-molecular-weight proteins utilize selective incorporation of protons and ^{13}C isotopes into methyl groups of Ile$^{\delta1}$, Leu$^{\delta}$ and Val$^{\gamma}$ side-chains in a highly deuterated environment (commonly referred to as "ILV labeling") providing a large number of high-quality probes for NMR studies of protein structure and dynamics. Robust ILV labeling methods[1,2] and strategic location of ILV side-chains in the hydrophobic cores of protein structures[3,4] have turned ILV labeling into an indispensable tool for NMR studies of large proteins and macromolecular assemblies.[5]

More than a decade ago, Gardner & Kay pioneered a technique for selective incorporation of protonated Ile$^{\delta1}$ methyl positions into protein molecules.[6] Subsequently, selective methyl labeling/protonation methods have been refined and extended to Leu$^{\delta}$ and Val$^{\gamma}$ methyl sites by the Kay group.[7,8] The introduction of methyl transverse relaxation optimized spectroscopy[9] (methyl-TROSY) has stimulated the development of improved labeling schemes.[10,11] Selective labeling of ILV methyl sites in large proteins on a deuterated background in synergy with methyl-TROSY techniques have had a significant impact on NMR studies of structure and dynamics of large protein assemblies up to ~800 kDa in molecular weight.[12-15] Nevertheless, the availability of only three ILV probes oftentimes presents a serious limitation for structural and dynamics studies of large proteins. Recent advances in methyl isotope labeling have focused on the extension of the ILV labeling methodology to methyl positions of alanines (Ala$^{\beta}$).

Methyl groups of alanines serve as an attractive extension of the ILV labeling methodology. Ala methyls are located in close proximity to the backbone; their flexibility is therefore reduced compared to other methyl-bearing side-chains in proteins. Ala is one of the most frequently encountered residues in protein hydrophobic cores and at molecular interfaces.[16] Recently, several reports have focused upon extending the ILV methodology with (selective) labeling of Ala$^{\beta}$ methyl positions in large proteins. Isaacson *et al.* have introduced a labeling strategy for selective incorporation of $^{13}CH_3$ groups into alanine residues on a deuterated background.[17] Specific ^{13}C labeling and protonation of Ala$^{\beta}$ methyls to a level of 95% with minimal background labeling (<1%) in minimal D_2O-based bacterial medium supplemented with large amounts of selectively ^{13}C-labeled α-deuterated alanine and co-addition of three deuterated compounds: (i) α-ketoisovalerate-D_7, (ii) succinate-D_4 and (iii) L-isoleucine-D_{10} has been reported by Boisbouvier and coworkers.[18] This labeling protocol has been closely followed for production of {^{2}H; Ala$^{\beta}$-[$^{13}CH_3$]}-labeled Malate Synthase G[19,20] (MSG) – an 82-kDa enzyme containing 73 methyl groups. Alanine is the most abundant residue in MSG comprising 10.1% of the total amino-acid content. Figure 1 shows the methyl-TROSY ^{1}H-^{13}C correlation map of MSG prepared using the protocol described in more detail in Appendix 1.

To maximize the number of available methyl probes, it is clearly advantageous to combine selective labeling of Ala positions with ILV methyl labeling.[21] The use of selectively $^{13}CH_3$-labeled Ala$^{\beta}$ ({2-D, 3-^{13}C}-L-alanine) in combination with (i) selectively $^{13}CH_3$-labeled α-ketobutyrate for labeling of Ile$^{\delta1}$ poistions, (ii) $^{13}CH_3$/$^{12}CD_3$-labeled α-ketoisovalerate for labeling of Val$^{\gamma}$ and Leu$^{\delta}$ sites, and (iii) deuterated succinate, achieves exactly this purpose (see Appendix 1 for the details about the used compounds). Figure 2 shows the ^{1}H-^{13}C methyl-TROSY correlation map recorded on the {Ala$^{\beta}$-[$^{13}CH_3$]; Ile$^{\delta1}$-[$^{13}CH_3$]; Leu,Val-[$^{13}CH_3$/$^{12}CD_3$]}-labeled MSG. Although the overlap of resonances within the Ala$^{\beta}$ group is very substantial, in MSG Ala$^{\beta}$ signals do not overlap with either Val$^{\gamma}$ or Leu$^{\delta}$ methyl groups.

In full agreement with previous observations,[18] no signs of scrambling of isotope labels from alanine to other amino-acids have been detected in either sample. In fact, a comparison of signal intensities of ILV methyls in the {U-^{2}H; Ala$^{\beta}$-[$^{13}CH_3$]; Ile$^{\delta1}$-[$^{13}CH_3$]; Leu,Val-[$^{13}CH_3$/$^{12}CD_3$]}-labeled sample with those obtained in {U-^{2}H; Ile$^{\delta1}$-[$^{13}CH_3$]; Leu,Val-[$^{13}CH_3$/$^{12}CD_3$]}-labeled MSG (without Ala$^{\beta}$ labeling) indicated that the levels of isotope incorporation into ILV methyl positions are not compromised to any significant extent by additions of large amounts of labeled Ala to the medium. This is the direct consequence of the fact that the biosynthetic pathway leading to incorporation of labels into Ile$^{\delta1}$ positions is "short-circuited" by addition of the suitably labeled α-ketobutyrate to the medium, while the pathway leading to labeling of Val$^{\gamma}$ and Leu$^{\delta}$ sites is "short-circuited" by addition of the α-keto-isovalerate precursor. It might have been expected that the addition of α-ketobutyrate to the medium instead of deuterated isoleucine in order to ensure $^{13}CH_3$ labeling at Ile$^{\delta1}$ methyl positions, would result in partial $^{13}CH_3$ labeling at Ile$^{\gamma2}$ methyl sites (arising from alanine-derived [3-$^{13}CH_3$]-pyruvate entering into the biosynthetic cycle of Ile$^{\delta}$ instead of the completely deuterated pyruvate from

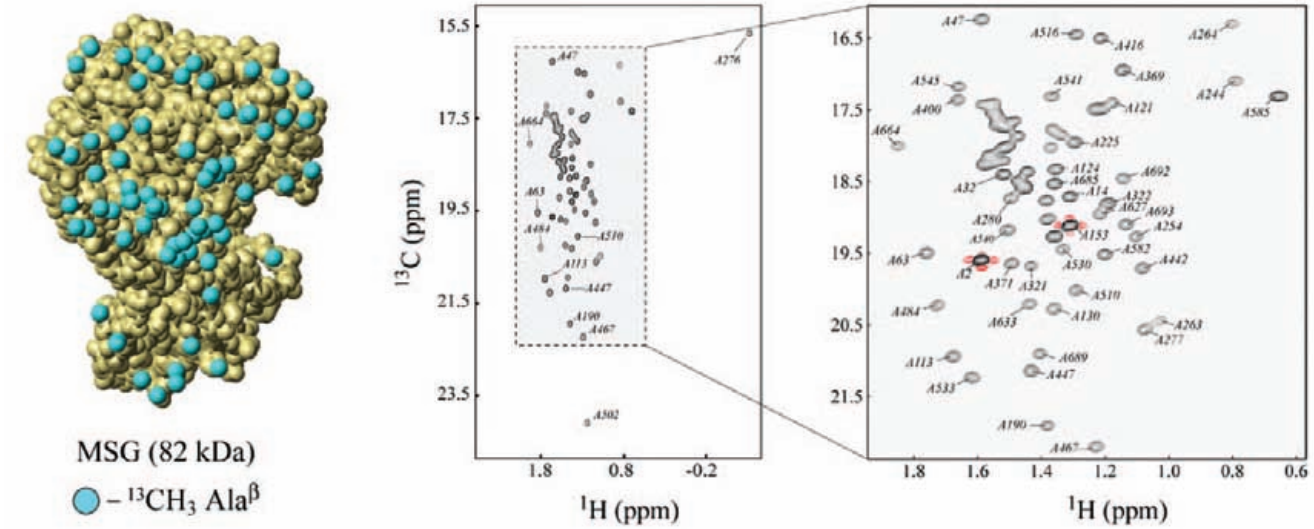

Figure 1. Left: Schematic representation of the structure of Malate Synthase G (82-kDa; 73 alanines) with positions of Ala$^{\beta}$ carbon atoms shown with cyan-colored spheres. Center: Methyl ^{1}H-^{13}C HMQC correlation map recorded on a 0.9 mM {U-^{2}H; Ala$^{\beta}$-[$^{13}CH_3$]}-labeled MSG (37°C; 600 MHz). Right: The "zoomed" part of the correlation map highlighted in the center. Selected assignments of separated Ala$^{\beta}$ cross-peaks are indicated.[22] Negative contours are shown in red.

[U-^{2}H]-glucose). Nevertheless, Ile$^{\gamma 2}$ ^{1}H-^{13}C correlations have not been detected in the ^{1}H-^{13}C methyl-TROSY spectra recorded on the {Ala$^{\beta}$-[$^{13}CH_3$]; Ile$^{\delta 1}$-[$^{13}CH_3$]; Leu,Val-[$^{13}CH_3$ / $^{12}CD_3$]}-MSG indicating that during protein production the molecules of pyruvate originate predominantly from [U-^{2}H]-glucose.

Proximity of alanine side-chains to the protein backbone and their high degree of order turn Ala$^{\beta}$ methyl groups into excellent NMR probes for a number of applications: (i) measurements of ^{1}H-^{13}C residual dipolar couplings (RDCs) in Ala$^{\beta}$ methyls, (ii) characterization of fast (pico-to-nanosecond) and slow (µs-to-millisecond) dynamics at functionally important sites of enzymes, and (iii) methyl-TROSY NOE spectroscopy that can be performed on {U-^{2}H; Ala$^{\beta}$–[$^{13}CH_3$]; Ile$^{\delta 1}$-[$^{13}CH_3$]; Leu, Val-[$^{13}CH_3$ / $^{12}CD_3$]}-labeled samples increasing the number of methyl probes for derivation of distance restraints compared to conventional ILV labeling methodology.

Appendix 1. Selective labeling of Ala$^{\beta}$ positions with $^{13}CH_3$ groups (the {U-[^{2}H]; Ala$^{\beta}$-[$^{13}CH_3$]}-labeled sample) has been achieved following the protocol of Ayala *et al.*[18] using [U-^{2}H]-D-glucose as the main carbon source in *E.coli* medium and addition of (i) 800 mg of {2-D, 3-^{13}C}-L-alanine, (ii) 2.5g of succinate-D_4, (iii) 200 mg for α-keto-isovalerate-D_7 and (iv) 60 mg of L-isoleucine-D_{10} to 1 liter of D_2O-based M9 medium one hour prior to induction of protein overexpression with 1 mM IPTG. Selective $^{13}CH_3$ labeling of all ILV positions together with Ala$^{\beta}$ methyls ({U-[^{2}H]; Ala$^{\beta}$-[$^{13}CH_3$]; Ile$^{\delta 1}$-[$^{13}CH_3$]; Leu,Val-[$^{13}CH_3$ / $^{12}CD_3$]}-labeled sample) has been achieved using the same carbon sources as above by addition of (i) 800 mg of {2-D,3-^{13}C}-L-alanine; (ii) 2.5g of succinate-D_4; (iii) 120 mg of α-ketoisovaleric acid, sodium salt (3-methyl-^{13}C: 3,4,4,4-D_4) and (iv) 60 mg of α-Ketobutyric acid, sodium salt (3-methyl-^{13}C; 3,3-D_2) to 1 liter of the medium 1 hour prior to induction. {Ala$^{\beta}$-[$^{13}CH_3$]}- and {Ala$^{\beta}$-[$^{13}CH_3$]; Ile$^{\delta 1}$-[$^{13}CH_3$]; Leu,Val-[$^{13}CH_3$ / $^{12}CD_3$]}-labeled samples of MSG were 0.9 mM and 0.75 mM in protein concentration, respectively, in 99% D_2O and contained 25 mM sodium phosphate buffer (pH 7.1, uncorrected), 20 mM $MgCl_2$, 5 mM DTT and 0.05% NaN_3.

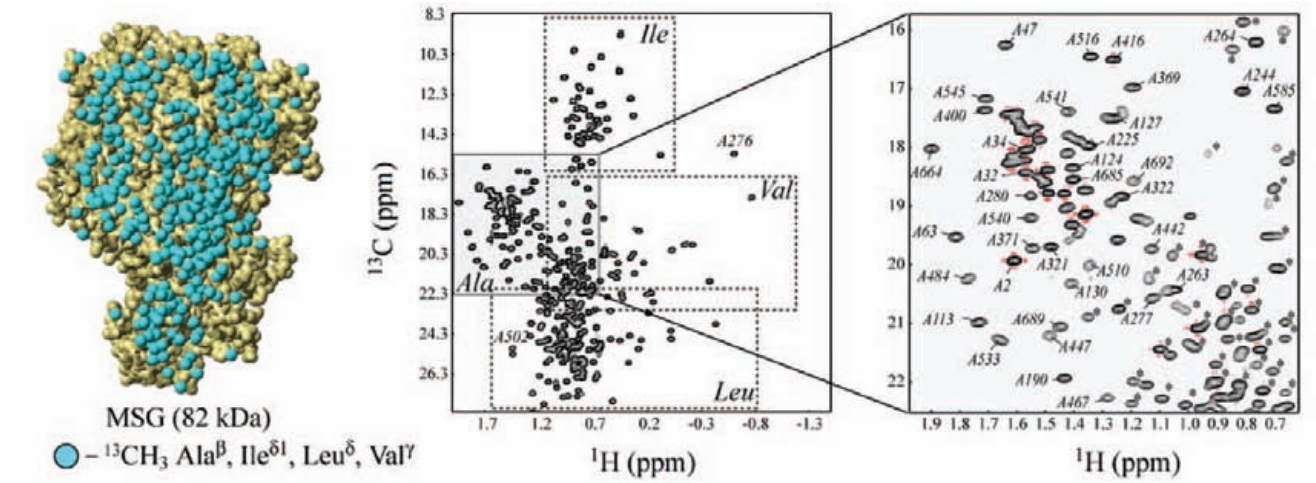

Figure 2. Left: Schematic representation of the structure of MSG with methyl carbons of Ala$^{\beta}$, Ile$^{\delta 1}$, Leu$^{\delta}$ and Val$^{\gamma}$ positions shown with cyan-colored spheres. Center: Methyl ^{1}H-^{13}C HMQC correlation map recorded on a 0.75 mM {Ala$^{\beta}$-[$^{13}CH_3$]; Ile$^{\delta 1}$-[$^{13}CH_3$]; Leu,Val-[$^{13}CH_3$ / $^{12}CD_3$]}-labeled MSG (37 °C; 600 MHz). The regions of the map approximately corresponding to Ile$^{\delta 1}$, Val$^{\gamma}$ and Leu$^{\delta}$ methyl positions are enclosed in dashed rectangles. The region enclosed in a solid rectangle and highlighted corresponds to Ala$^{\beta}$ methyl correlations. Right: The Ala$^{\beta}$ part of the map "zoomed" from the region highlighted in the center. Cross-peaks arising from Val$^{\gamma}$ and Leu$^{\delta}$ correlations are marked with asterisks. Ala$^{\beta}$ assignments are indicated for selected methyls.[22]

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CDLM-7317	α-Ketoisovaleric acid, sodium salt (3-methyl-^{13}C, 99%; 3,4,4,4-D_4, 98%)	0.12 g
CDLM-7318	α-Ketobutyric acid, sodium salt (3-methyl-^{13}C, 99%; 3,3-D_2, 98%)	0.06 g
CDLM-8649	L-Alanine (2-D, 99%; 3-^{13}C, 96%)	0.8 g

The materials in this kit are to be used in conjunction with 1 L of deuterated minimal media. 1 L of minimal media that is suitable for use with this product typically contains 2 g of glucose-D_7, 1 g of ammonium salt (either ^{15}N labeled or unlabeled, depending on preference of the user), and 11.3 g of M9 salts in D_2O.

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The materials in this kit are to be used in conjunction with 1 L of deuterated minimal media.[1,2] 1 L of minimal media that is suitable for use with this product typically contains 2 g of glucose-D_7, 1 g of ammonium salt (either ^{15}N labeled or unlabeled, depending on preference of the user), and 11.3 g of M9 salts in D_2O.

[1] Ayala, I.; Sounier, R.; Use, N.; Gans, P.; Boisbouvier, J. **2009**. *J Biomol NMR, 43,* 111-9.

[2] Isaacson, R.L.; Simpson, P.J.; Liu, M.; Cota, E.; Zhange, X.; Freemont, P.; Matthews, S. **2007**. *J Am Chem Soc, 129,* 15428-9.

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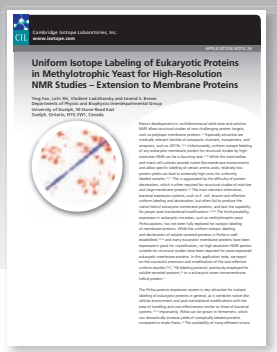
References

1. Tugarinov, V.; Kanelis, V.; Kay, L.E. **2006**. *Nat Protocols, 1*, 749.
2. Sprangers, R.; Velyvis, A.; Kay, L.E. **2007**. *Nat Methods, 4*, 697.
3. Gardner, K.H.; Rosen, M.K.; Kay, L.E. **1997**. *Biochemistry, 36*, 1389.
4. Rosen, M.K.; Gardner, K.H.; Willis, R.C.; Parris, J.E.; Pawson, T.; Kay, L.E. **1996**. *J Mol Biol, 263*, 627.
5. Tugarinov, V.; Hwang, P.M.; Kay, L.E. **2004**. *Annu Rev Biochem, 73*, 107.
6. Gardner, K.H.; Kay, L.E. **1997**. *J Am Chem Soc, 119*, 7599.
7. Gardner, K.H.; Zhang, X.; Gehring, K.; Kay, L.E. **1998**. *J Am Chem Soc, 120*, 11738.
8. Goto, N.K.; Gardner, K.H.; Mueller, G.A.; Willis, R.C.; Kay, L.E. **1999**. *J Biomol NMR, 13*, 369.
9. Tugarinov, V.; Hwang, P.M.; Ollerenshaw, J.E.; Kay, L.E. **2003**. *J Am Chem Soc, 125*, 10420.
10. Tugarinov, V.; Kay, L.E. **2004**. *J Biomol NMR, 28*, 165.
11. Gans, P.; Hamelin, O.; Sounier, R.; Ayala, I.; Durá, M.A.; Amero, C.D.; Noirclerc-Savoye, M.; Franzetti, B.; Plevin, M.J.; Boisbouvier, J. **2010**. *Angew Chem Int Ed Engl, 49*, 1958.
12. Tugarinov, V.; Choy, W.Y.; Orekhov, V.Y.; Kay, L.E. **2005**. *Proc Natl Acad Sci USA, 102*, 622.
13. Sprangers, R.; Kay, L.E. **2007**. *Nature, 445*, 618.
14. Religa, T.L.; Sprangers, R.; Kay, L.E. **2010**. *Science, 328*, 98.
15. Velyvis, A.; Yang, Y.R.; Schachman, H.K.; Kay, L.E. **2007**. *Proc Natl Acad Sci USA, 104*, 8815.
16. Fernandez, A.; Scott, L.R.; Scheraga, H.A. **2003**. *J Phys Chem, 107*, 9929.
17. Isaacson, R.L.; Simpson, P.J.; Liu, M.; Cota, E.; Zhang, X.; Freemont, P.; Matthews, S. **2007**. *J Am Chem Soc, 129*, 15428.
18. Ayala, I.; Sounier, R.; Usé, N.; Gans, P.; Boisbouvier, J. **2009**. *J Biomol NMR, 43*, 111.
19. Howard, B.R.; Endrizzi, J.A.; Remington, S. **2000**. *J Biochemistry, 39*, 3156.
20. Tugarinov, V.; Muhandiram, R.; Ayed, A.; Kay, L.E. **2002**. *J Am Chem Soc, 124*, 10025.
21. Godoy-Ruiz, R.; Guo, C.; Tugarinov V. **2010**. *J Am Chem Soc, 132*(51) 18340.
22. Sheppard, D.; Guo, C.; Tugarinov, V. **2009**. *J Am Chem Soc, 131*, 1364.



Uniform Isotope Labeling of Eukaryotic Proteins in Methylotrophic Yeast for High-Resolution NMR Studies – Extension to Membrane Proteins

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Recent developments in multidimensional solid-state and solution NMR allow structural studies of new challenging protein targets, such as polytopic membrane proteins.[1-3] Especially attractive are medically relevant families of eukaryotic channels, transporters, and receptors, such as GPCRs.[1,4-6] Unfortunately, uniform isotope labeling of any eukaryotic membrane protein for structural studies by high-resolution NMR can be a daunting task.[7-10] While the mammallian and insect cell cultures provide native-like membrane environments and allow specific labeling of certain amino acids, relatively low protein yields can lead to extremely high costs for uniformly labeled samples.[10,11] This is aggravated by the difficulty of protein deuteration, which is often required for structural studies of mid-size and large membrane proteins.[10] The most common alternative, bacterial expression systems, such as *E. coli*, ensure cost-effective uniform labeling and deuteration, but often fail to produce the native fold of eukaryotic membrane proteins, and lack the capability for proper post-translational modifications.[1,6,8,9] The third possibility, expression in eukaryotic microbes, such as methylotrophic yeast *Pichia pastoris*, has not been fully explored for isotopic labeling of membrane proteins. While the uniform isotopic labeling and deuteration of soluble secreted proteins in *Pichia* is well-established,[12-16] and many eucaryotic membrane proteins have been expressed in yeast for crystallization, no high-resolution NMR spectra suitable for structural studies have been reported for yeast-expressed eukaryotic membrane proteins. In this application note, we report on the successful extension and modification of the cost-effective uniform double (^{13}C,^{15}N) labeling protocol, previously employed for soluble secreted proteins,[16] to a eukaryotic seven-transmembrane helical protein.[17]

The *Pichia pastoris* expression system is very attractive for isotopic labeling of eukaryotic proteins in general, as it combines native-like cellular environment and post-translational modifications with the ease of handling and cost-effectiveness similar to those of bacterial systems.[10,14] Importantly, *Pichia* can be grown in fermenters, which can dramatically increase yields of isotopically labeled proteins compared to shake flasks.[13] The availability of many different strains and vectors allow optimization of selection of best transformants, cellular targeting, and protein tagging for specific proteins.

The double (^{13}C,^{15}N) uniform isotope labeling of soluble secreted proteins in *Pichia* relies on a very straightforward protocol, in which the carbon source at the pre-induction growth phase (*e.g.* $^{13}C_6$-glucose) is replaced by ^{13}C-methanol in the post-induction phase.[16] Concentrations and timing of addition of isotope sources have been optimized to ensure the cost-effectiveness and completeness of labeling.[14] In view of high-yield functional expression of many non-labeled mammalian membrane proteins, for example, aquaporins and GPCRs,[18-20] in *Pichia*, it is logical to adapt the existing isotope-labeling protocols for these attractive targets.

We have demonstrated the feasibility of such an approach[17] by conducting cost-efficient uniform ^{13}C,^{15}N-labeling of a ~31 kDa eukaryotic membrane protein, *Leptosphaeria* rhodopsin (LR),[21,22] which shares seven-transmembrane architecture with GPCRs. The *lop* gene encoding N-terminally truncated, C-terminally 6-His-tagged LR was placed in pPICZαA vector (Invitrogen), which allowed for selection of multiple integration events on zeocin plates and efficient targeting of the protein-to-plasma membrane using the secretion signal (α-factor). The expression was conducted using the protease-deficient strain SMD1168H (Invitrogen) in shake flasks at 30ºC. The pre-induction growth was performed in 250 mL of ^{13}C,^{15}N-BMD (buffered minimal dextrose) with 0.5% $^{13}C_6$-glucose and 0.8% $^{15}NH_4Cl$, while the post-induction growth was done in 0.8 L of ^{13}C,^{15}N-BMM (buffered minimal methanol) with 0.5% ^{13}C-methanol and 0.8% $^{15}NH_4Cl$. ^{13}C-methanol was added to the growth medium again after 24 hours of induction, to the final concentration of 0.5%, along with all-*trans*-retinal chromophore needed to regenerate the opsin. After the small-scale screening for the best-producing colonies and optimization of the harvesting time (40 hours after the induction; shorter and longer times gave substantially lower yields), the yield of the purified protein exceeded 5 mg per liter of culture. As we used only 0.5% concentrations of ^{13}C-glucose and methanol, and the latter had to be replenished only once, the cost of this sample is close to that for similar bacterial proteins produced in *E. coli*.[23,24]

After breaking the lyticase-treated cells with glass beads, and solubilizing the membranes with Triton X-100, the protein was purified using Ni^{2+}-NTA 6-His-tag affinity resin (Qiagen). At this

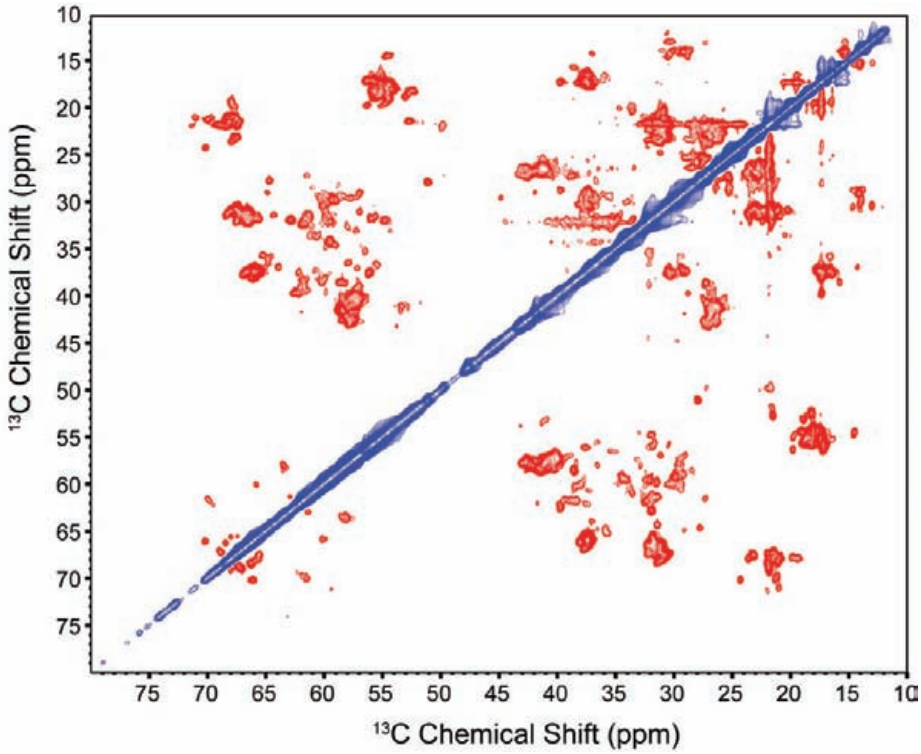


Figure 1. Two-dimensional carbon-carbon chemical shift correlation spectrum of the [U-^{13}C,^{15}N] labeled LR at 800 MHz proton frequency with 1.26 ms of SPC5$_3$ mixing.[26] Spinning frequency was 14.3 kHz. Acquisition lengths were 11 ms in t_1 and 20.48 ms in t_2 acquisition dimensions.

stage, the purity and homogeneity of solubilized LR were estimated spectrophotometrically, by SDS PAGE, and by MALDI TOF mass spectrometry. We found that LR was pure, lacked glycosylation, had the secretion signal cleaved off (with the exception of the last four residues), and had a high extent of isotopic labeling. The extent of isotopic labeling and functionality of the expressed protein were further explored by static and time-resolved Fourier-transform infrared (FTIR) spectroscopy,[17,25] after reconstitution into the membrane-mimicking DMPC/DMPA liposomes. Static FTIR spectra of the dry proteoliposome films confirmed the high (>90%) overall extent of both ^{13}C and ^{15}N labeling, while time-resolved FTIR spectroscopy of hydrated liposomes showed fully functional photochemistry and proton transfers, along with complete isotope labeling of specific sidechains. The samples were stable for at least several weeks when kept at 4°C.

Magic angle spinning solid-state NMR measurements, conducted on hydrated LR proteoliposomes using 600 MHz and 800 MHz Bruker instruments, confirmed high structural homogeneity and purity of the sample, along with very low extent of glycosylation. Two-dimensional ^{13}C-^{13}C SPC-5$_3$[26] and NCOCX correlation spectra (Figures 1 and 2) show many well-resolved resonances with estimated spectral linewidths of ~0.5 ppm for ^{13}C, 0.7 ppm for ^{15}N, on par with those observed in the best available samples of bacterial membrane proteins of similar size.[23,24,27,28] Such spectral resolution allows identification of individual resonances, and is sufficient for conducting spectroscopic assignments by multi-dimensional spectroscopy. Many peaks can be identified by the amino acid type (*e.g.*, prolines, alanines, threonines, serines). Resonances due to carboxylic acids could be resolved in previously published 2D ^{13}C-^{13}C DARR correlation spectra.[17] Furthermore, some resonances of prolines and protonated aspartates could be tentatively assigned based on the homology with bacterio-rhodopsin.[29,30] The peaks show high dispersion consistent with the co-existence of α-helical transmembrane regions and unstructured and β-stranded loops and tails. Integration of intensities of alanine and glycine peaks show that only a small portion of the resonances are not observed in the 2D ^{13}C-^{13}C spectra, some of which may not be detectable because of unfavorable dynamics in the long unstructured loops and the C-tail.

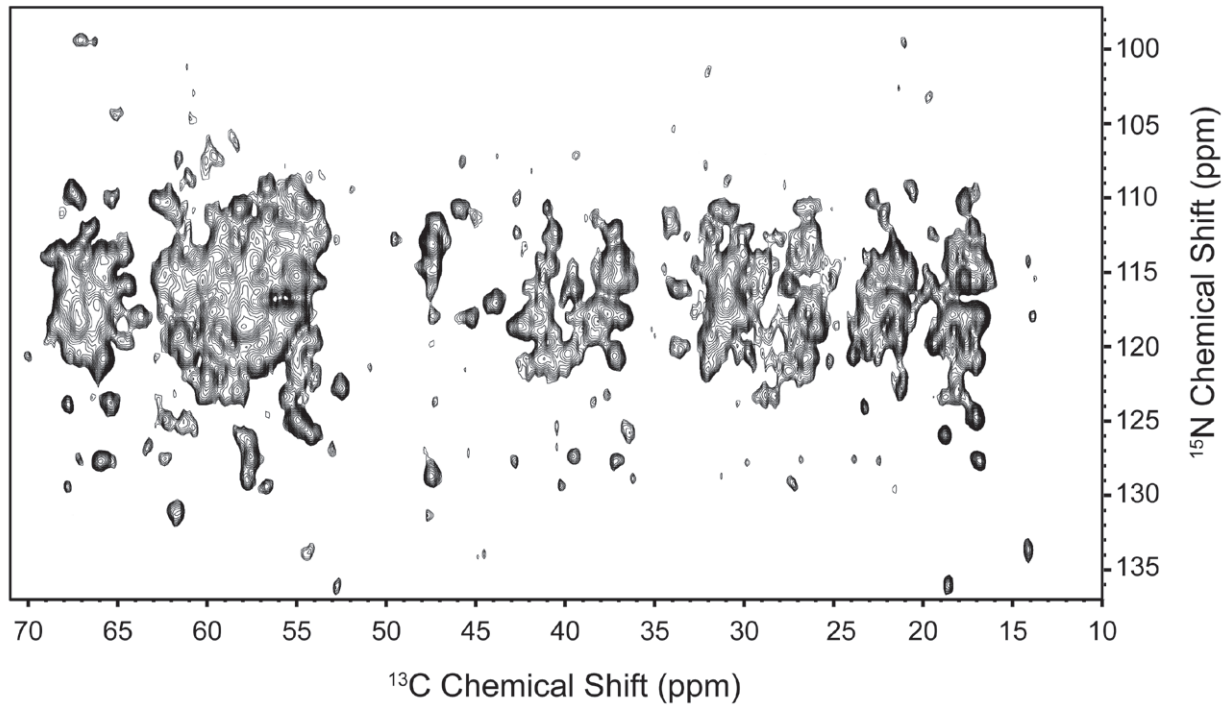


Figure 2. Aliphatic region of two-dimensional NCOCX chemical shift correlation experiment recorded at 600 MHz (proton), 12.5 kHz spinning frequency, and with 30 ms of DARR carbon-carbon mixing. Acquisition lengths were 15 ms in t_1 and 22.3 ms in t_2 acquisition dimensions.

In summary, we have demonstrated that cost-effective uniform ^{13}C,^{15}N labeling of mid-size eukaryotic membrane proteins can be realized in *Pichia pastoris*, using ^{13}C-methanol as a main carbon source. The produced samples are homogeneous, functional, stable, and yield high-resolution solid-state NMR spectra suitable for structural studies. We hope that similar protocols can be adopted for challenging mammalian membrane proteins of medical relevance.

References

1. Kim, H.J.; Howell, S.C.; Van Horn, W.D.; Jeon, Y.H.; Sanders, C.R. **2009**. *Prog Nucl Magn Reson Spectrosc, 55*, 335.
2. Gautier, A.; Mott, H.R.; Bostock, M.J.; Kirkpatrick, J.P.; Nietlispach, D. **2010**. *Nat Struct Mol Biol, 17*, 768.
3. Renault, M.; Cukkemane, A.; Baldus, M. **2010**. *Angew Chem Int Ed Engl, 49*, 8346.
4. Tikhonova, I.G.; Costanzi, S. **2009**. *Curr Pharm Des, 15*, 4003.
5. Goncalves, J.A.; Ahuja, S.; Erfani, S.; Eilers, M.; Smith, S.O. **2010**. *Prog Nucl Magn Reson Spectrosc, 57*, 159.
6. Tapaneeyakorn, S.; Goddard, A.D.; Oates, J.; Willis, C.L.; Watts, A. **2011**. *Biochim Biophys Acta,* in press.
7. Lundstrom, K.; Wagner, R.; Reinhart, C.; Desmyter, A.; Cherouati, N.; Magnin, T.; Zeder-Lutz, G.; Courtot, M.; Prual, C.; Andre, N.; Hassaine, G.; Michel, H.; Cambillau, C.; Pattus, F. **2006**. *Journal of structural and functional genomics, 7*, 77.
8. Sarramegna, V.; Muller, I.; Milon, A.; Talmont, F. **2006**. *Cellular and Molecular Life Sciences, 63*, 1149.
9. McCusker, E.C.; Bane, S.E.; O'Malley, M.A.; Robinson, A.S. **2007**. *Biotechnol Prog*, *23*, 540.
10. Takahashi, H.; Shimada, I. **2010**. *J Biomol NMR, 46*, 3.
11. Egorova-Zachernyuk, T.A.; Bosman, G.J.; Degrip, W.J. **2011**. *Appl Microbiol Biotechnol*, *89*, 397.
12. Laroche, Y.; Storme, V.; De Meutter, J.; Messens, J.; Lauwereys, M. **1994**. *Biotechnology* (NY), *12*, 1119.
13. Wood, M.J.; Komives, E.A. **1999**. *J Biomol NMR, 13*, 149.
14. Rodriguez, E.; Krishna, N.R. **2001**. *J Biochem, 130*, 19.
15. Morgan, W.D.; Kragt, A.; Feeney, J. **2000**. *J Biomol NMR, 17*, 337.
16. Pickford, A.R.; O'Leary, J.M. **2004**. *Methods in molecular biology (Clifton, NJ), 278*, 17.
17. Fan, Y.; Shi, L.; Ladizhansky, V.; Brown, L.S. **2011**. *J Biomol NMR, 49,* 151-161.
18. Oberg, F.; Ekvall, M.; Nyblom, M.; Backmark, A.; Neutze, R.; Hedfalk, K. **2009**. *Mol Membr Biol, 26*, 215.
19. Singh, S.; Hedley, D.; Kara, E.; Gras, A.; Iwata, S.; Ruprecht, J.; Strange, P.G.; Byrne, B. **2010**. *Protein Expr Purif, 74*, 80.
20. Andre, N.; Cherouati, N.; Prual, C.; Steffan, T.; Zeder-Lutz, G.; Magnin, T.; Pattus, F.; Michel, H.; Wagner, R.; Reinhart, C. **2006**. *Protein Sci, 15*, 1115.
21. Idnurm, A.; Howlett, B. J. **2001**. *Genome, 44*, 167.
22. Waschuk, S.A.; Bezerra, A.G.; Shi, L.; Brown, L.S. **2005**. *P Natl Acad Sci USA, 102*, 6879.
23. Shi, L.; Ahmed, M.A.; Zhang, W.; Whited, G.; Brown, L.S.; Ladizhansky, V. **2009**. *J Mol Biol, 386*, 1078.
24. Shi, L.; Kawamura, I.; Jung, K.H.; Brown, L.S.; Ladizhansky, V. **2011**. *Angew Chem Int Ed Engl, 50*, 1302.
25. Egorova-Zachernyuk, T.A.; Bosman, G.J.; Pistorius, A.M.; DeGrip, W.J. **2009**. *Appl Microbiol Biotechnol, 84*, 575.
26. Hohwy, M.; Rienstra, C.M.; Griffin, R.G. **2002**. *J Chem Phys, 117*, 4973.
27. Etzkorn, M.; Martell, S.; Andronesi, O.C.; Seidel, K.; Engelhard, M.; Baldus, M. **2007**. *Angewandte Chemie-International Edition, 46*, 459.
28. Varga, K.; Aslimovska, L.; Watts, A. **2008**. *J Biomol NMR, 41*, 1.
29. Lansing, J.C.; Hu, J.G.G.; Belenky, M.; Griffin, R.G.; Herzfeld, J. **2003**. *Biochemistry-Us, 42*, 3586.
30. Metz, G.; Siebert, F.; Engelhard, M. **1992**. *Biochemistry-Us, 31*, 455.

NMR Solvent Data Chart

More Solvents, More Sizes, More Solutions

	^{1}H Chemical Shift (ppm from TMS) (multiplicity) ●	JHD (Hz)	^{13}C Chemical Shift (ppm from TMS) (multiplicity) ●	JCD (Hz)	^{1}H Chemical Shift of HOD (ppm from TMS) ❑	Density at 20°C ◆	Melting point (°C) ◆	Boiling point (°C) ◆	Dielectric Constant	Molecular Weight ◆
Acetic Acid-d_4	11.65 (1) 2.04 (5)	2.2	178.99 (1) 20.0 (7)	20	11.5	1.12	16.7	118	6.1	64.08
Acetone-d_6	2.05 (5)	2.2	206.68 (1) 29.92 (7)	0.9 19.4	2.8*	0.87	-94	56.5	20.7	64.12
Acetonitrile-d_3	1.94 (5)	2.5	118.69 (1) 1.39 (7)	21	2.1*	0.84	-45	81.6	37.5	44.07
Benzene-d_6	7.16 (1)		128.39 (3)	24.3	0.4	0.95	5.5	80.1	2.3	84.15
Chloroform-d	7.24 (1)		77.23 (3)	32.0	1.5*	1.50	-63.5	61-62	4.8	120.38
Cyclohexane-d_{12}	1.38 (1)		26.43 (5)	19	0.8	0.89	6.47	80.7	2.0	96.24
Deuterium Oxide	4.80 (DSS) 4.81 (TSP)		NA	NA	4.8	1.11	3.81	101.42	78.5	20.03
N, N-Dimethyl-formamide-d_7	8.03 (1) 2.92 (5) 2.75 (5)	 1.9 1.9	163.15 (3) 34.89 (7) 29.76 (7)	29.4 21.0 21.1	3.5	1.03	-61	153	36.7	80.14
Dimethyl Sulfoxide-d_6	2.50 (5)	1.9	39.51 (7)	21.0	3.3*	1.19	18.55	189	46.7	84.17
1,4-Dioxane-d_8	3.53 (m)		66.66 (5)	21.9	2.4	1.13	11.8	101.1	2.2	96.16
Ethanol-d_6	5.19 (1) 3.56 (1) 1.11 (m)		 56.96 (5) 17.31 (7)	 22 19	5.3	0.89	-114.1	78.5	24.5	52.11
Methanol-d_4	4.78 (1) 3.31 (5)	 1.7	 49.15 (7)	 21.4	4.9	0.89	-97.8	64.7	32.7	36.07
Methylene Chloride-d_2	5.32 (3)	1.1	54.00 (5)	27.2	1.5	1.35	-95	39.75	8.9	86.95
Pyridine-d_5	8.74 (1) 7.58 (1) 7.22 (1)		150.35 (3) 135.91 (3) 123.87 (3)	27.5 24.5 25	5	1.05	-41.6	115.2-115.3	12.4	84.13
1,1,2,2-Tetrachloroethane-d_2	6.0		73.78 (3)			1.62	-44	146.5	8.20	169.86
Tetrahydrofuran-d_8	3.58 (1) 1.73 (1)		67.57 (5) 25.37 (5)	22.2 20.2	2.4-2.5	0.99	-108.5	66	7.6	80.16
Toluene-d_8	 7.09 (m) 7.00 (1) 6.98 (5) 2.09 (5)	 2.3	137.86 (1) 129.24 (3) 128.33 (3) 125.49 (3) 20.4 (7)	 23 24 24 19	0.4	0.94	-95	110.6	2.4	100.19
Trifluoroacetic Acid-d	11.50 (1)		164.2 (4) 116.6 (4)		11.5	1.49	-15.4	72.4		115.03
Trifluoroethanol-d_3	5.02 (1) 3.88 (4x3)	 2(9)	126.3 (4) 61.5 (4x5)	 22	5	1.41	-43.5	74.05		103.06

M.J. O'Neil, P.E. Heckelman, C.B. Koch, K.J. Roman, *The Merck Index,* an Encyclopedia of Chemicals, Drugs, and Biologicals – Fourteenth Edition, Merck Co., Inc. Whitehouse Station, NJ 2006.

● The ^{1}H spectra of the residual protons and ^{13}C spectra were obtained on a Varian Gemini 200 spectrometer at 295°K. The NMR solvents used to acquire these spectra contain a maximum of 0.05% and 1.0% TMS (v/v) respectively. Since deuterium has a spin of 1, triplets arising from coupling to deuterium have the intensity ratio of 1:1:1. 'm' denotes a broad peak with some fine structures. It should be noted that chemical shifts can be dependent on solvent, concentration and temperature.

❑ Approximate values only; may vary with pH, concentration and temperature.

◆ Melting and boiling points are those of the corresponding unlabeled compound (except for D_2O). These temperature limits can be used as a guide to determine the useful liquid range of the solvents. Information gathered from the Merck Index – Fourteenth Edition.

* HOD Peaks – NMR spectra of "neat" deuterated solvent always exhibit a peak due to H_20 in addition to the residual solvent peak. When the exchange rate between H_20 and HDO is slow on the NMR timescale the water peak appears as two peaks, a singlet corresponding to H_20 and a 1:1:1 triplet corresponding to HDO.

NMR Solvent Storage and Handling Information

Please note that some packaging sizes of some solvents may require special handling not given below. The bottle or ampoule packaging information should be reviewed for further instructions.

Acetic Acid-d_4 / Acetone-d_6 / Benzene-d_6 / Cyclohexane-d_{12} / Deuterium Oxide / N,N-Dimethylformamide-d_7 / Dimethyl Sulfoxide-d_6 / 1,4-Dioxane-d_8 (p-Dioxane) / Ethanol-d_6 / Methanol-d_4 / Methylene Chloride-d_2 / Pyridine-d_5 / 1,1,2,2 Tetrachloroethane-d_2 / Toluene-d_8 / Trifluoroacetic Acid-d / 2,2,2-Trifluoroethanol-d_3

Store at room temperature away from light and moisture. The above products are stable if stored under recommended conditions.

Acetonitrile-d_3

Store at room temperature away from light and moisture. This product is stable for one year after receipt of order if stored under above conditions (unopened). After one year, the solvent should be re-analyzed for chemical purity before use.

Chloroform-d / Tetrahydrofuran-d_8

Store refrigerated (-5° to 5°C) away from light and moisture. These products are stable for six months after receipt of order if stored under above conditions (unopened). After six months, the solvent should be re-analyzed for chemical purity before use.

Deuterium Exchange of Labile Protons in Deuterated Solvents containing Residual D_2O

Some deuterated solvents are prepared by catalytic exchange of protonated solvent with deuterium oxide and carefully purified by distillation. Residual water (H_2O in equilibrium exchange with D_2O) is kept to a minimum of 20-200 ppm, the higher value corresponds to the amount in the more hygroscopic solvents. The labile deuterons (and protons) of water are available to exchange with labile protons in the chemist's sample and can result in inaccurate integration ratios. The example below shows that just 100 ppm of D_2O can cause problems when studying dilute solutions of analytes. A significant decrease in the integral of 1 labile proton may be observed in a sample containing 5 mg organic compound, MW~200, dissolved in 1g DMSO-d_6 containing 100 ppm D_2O. The problem becomes worse as the molecular weight of the analyte increases.

Solution

Water (as H_2O, HDO or D_2O) can be minimized by adding molecular sieves to the solvent, agitating the mixture and allowing it to stand for a few hours. The water content may be reduced to about 10-20 ppm in this manner. If exchange still causes a problem, it is recommended to use a less hygroscopic solvent, such as chloroform, methylene chloride or acetonitrile.

X *– residual solvent* ******* *– residual water*

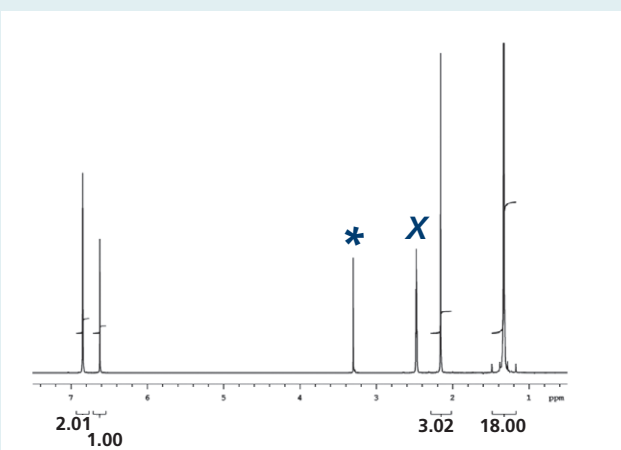


Figure 1. ^{1}H NMR spectrum of 5.0 mg 2,6-di-tert-butyl-4-methylphenol (MW 220.36g / mole) in dry DMSO-d_6. *Note the proper integral ratios of 18:3:1:2 (t-butyl: methyl: ring-H: -OH). Note the single H_2O peak at 3.3 ppm.*

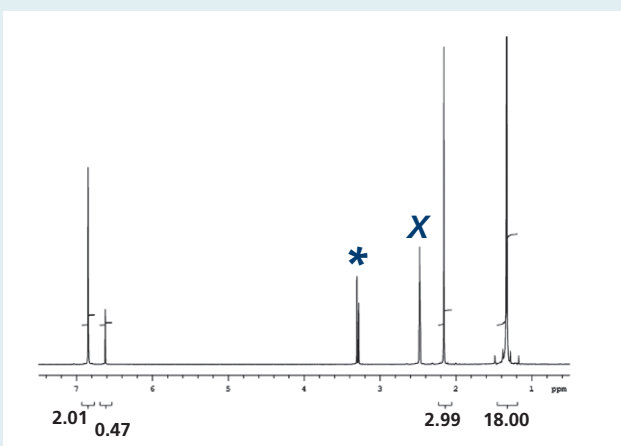
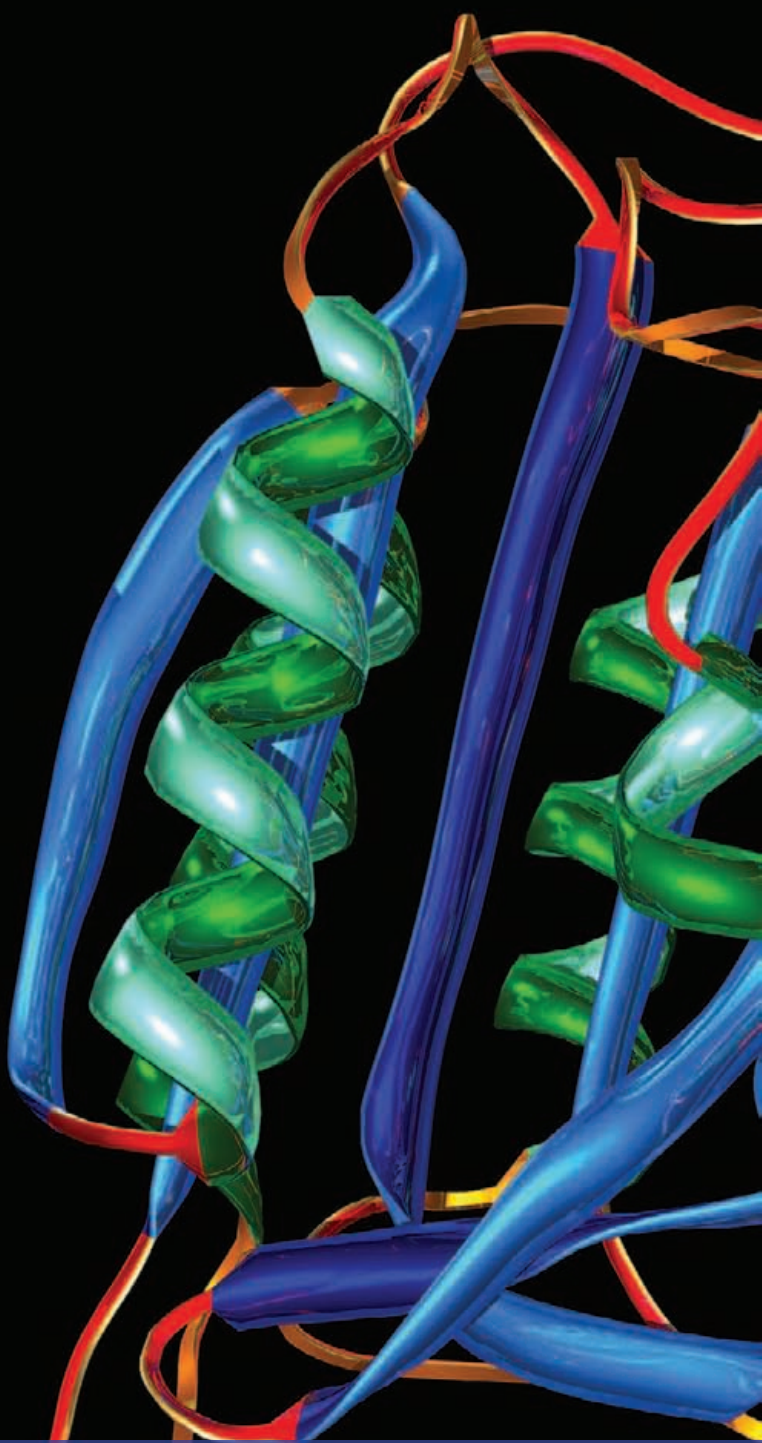


Figure 2. ^{1}H NMR spectrum of 5.3 mg of 2,6-di-tert-butyl-4-methylphenol in DMSO-d_6 with 100 ppm D_2O added. *Note the reduced ratio of the phenolic proton 18:3:2:0.47 (t-butyl: methyl: ring-H: -OH). Note that the HOH and HOD peaks are separated in the spectrum.*



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