



## Bioprocessing Tutorial

# HCDF as a Protein-Labeling Methodology

## Production of $^2\text{H}$ -, $^{13}\text{C}$ -, and $^{15}\text{N}$ -Labeled OmpG via High Cell Density Fermentation

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While NMR has proven to be an efficient technology for molecular imaging, it is also known for being expensive. This is related to the expensive labeled media required during the fermentation process. The study highlighted in this article shows that high cell density fermentation (HCDF) may provide an efficient alternative to traditional protein labeling methods.

$^2\text{H}$ -,  $^{13}\text{C}$ -,  $^{15}\text{N}$ -labeled outer-membrane protein G (DCN-OmpG) over-expressed in *E. coli* was needed for structural studies by solid-state (ss) magic-angle spinning (MAS) NMR. The protocol for production, purification, refolding, and reconstitution for NMR purposes and method development has been established at the FMP lab ([www.fmp-berlin.de](http://www.fmp-berlin.de)). Typically a 20-mg preparation starting from 2-L *E. coli* shaking culture grown to an  $\text{OD}_{600\text{nm}}$

of 2 is used to produce one sample for ssNMR. This implies the consumption of 2-L  $\text{D}_2\text{O}$  as well as the  $^{15}\text{N}$  and  $^{13}\text{C}$  isotopes for the preparation of one triple (DCN) labeled ssNMR-sample. To generate higher yields of labeled protein, Dasgip's ([www.dasgip.com](http://www.dasgip.com)) fermentation system fed batch pro<sup>®</sup> was adapted to produce recombinant protein for NMR analysis and other studies.

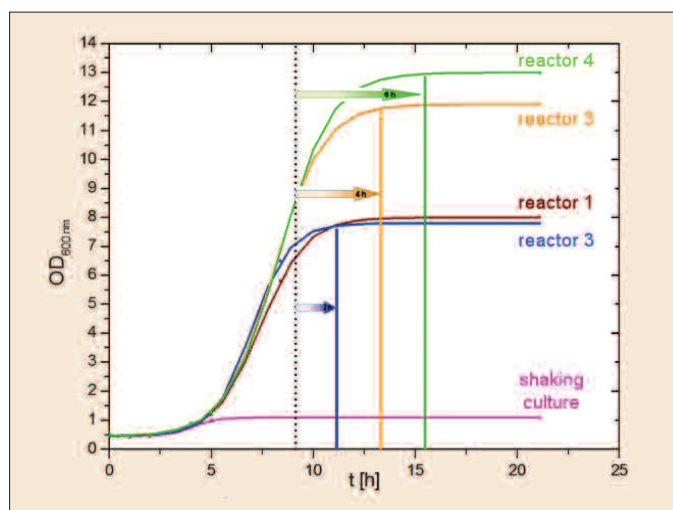
Dasgip bioreactor systems allow a fully automated HCDF process—growing the cells in the batch phase, starting

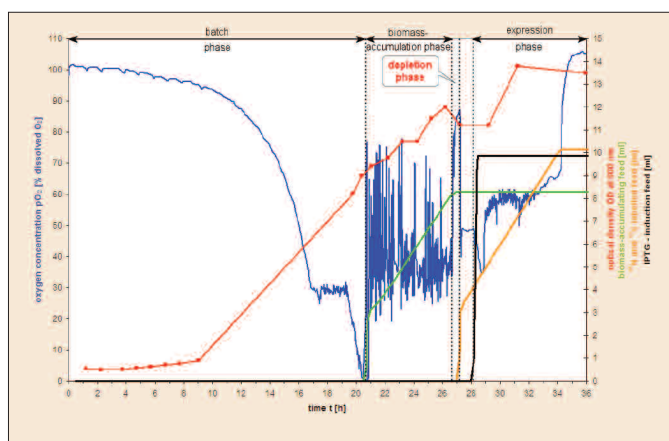
Sebastian Fiedler and Anne Diehl ([diehl@fmp-berlin.de](mailto:diehl@fmp-berlin.de)) are scientists and Hartmut Oschkinat is head of NMR at FMP. Christof Knocke is European sales manager and Jennefer Vogt is marketing and communications manager at Dasgip. Web: [www.dasgip.com](http://www.dasgip.com).

the fed-batch after the substrates have depleted, switching on the labeled feed 1 h before protein expression is induced, maintaining the feed during protein expression, then cooling down for harvest. Each step can be controlled with one system and can be integrated into one process.

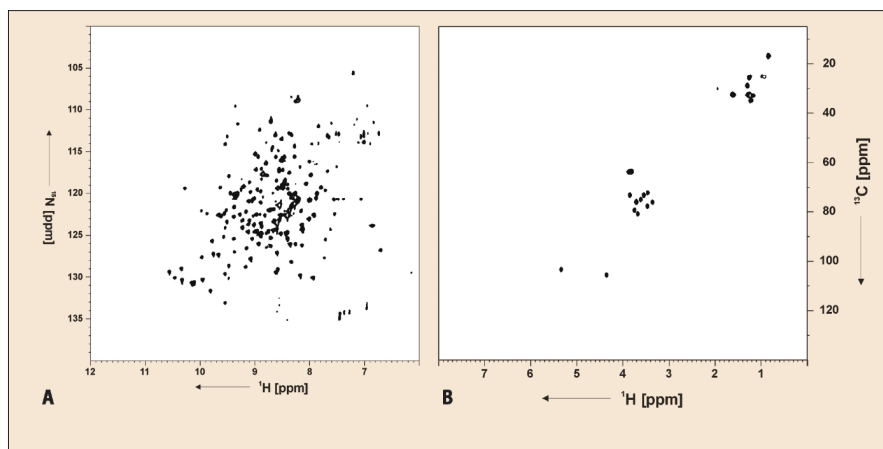
The *OmpG* gene was cloned into the

**Figure 1. Growth curves of four non-deuterated experiments to optimize the duration of the biomass accumulating feed (unlabeled, shown as arrows) from 0–6 h, followed by a labeled feed and protein expression in comparison to the shaking culture.**





**Figure 2. Graphical representation of selected parameters of the fermentation process on deuterated medium. Blue—DO, red—OD<sub>600nm</sub>, green—biomass accumulating feed (D<sub>2</sub>O but unlabeled with respect to N and C), yellow—labeled feed, and black—IPTG induction**



**Figure 3. Solution NMR spectra of <sup>2</sup>H<sup>13</sup>C<sup>15</sup>N-OmpG purified in water. (A) <sup>1</sup>H-<sup>15</sup>N correlation (HSQC-TROSY) shows a well-folded protein with back-exchanged protons. (B) <sup>1</sup>H-<sup>13</sup>C correlation (HMQC) only signals from the detergent are to be seen, as the deuterons bound to carbons give no signal in that spectrum and cannot be back-exchanged during purification.**

pET-26b vector (kanamycin resistance). The cloning procedure and all traditional protein expression and purification protocols were published previously.

For HCDF, Dasgip's bioreactor system was used with four parallel fermentation vessels, each contained 200-mL medium, and was programmed with the parameters in Table 1. A modified M9 minimal medium was used for the cultivation.

### Enhancing Cell Density

The expression experiments for OmpG using HCDF were performed on nondeuterated, unlabeled media for optimization. The batch medium contained sufficient substrates (8 g/L glucose, 2 g/L NH<sub>4</sub>Cl) for growing the *E. coli* to

OD<sub>600nm</sub> of about 8. Before induction, a biomass accumulating phase was introduced. First the effects of 0, 2, 4, and 6 h additional unlabeled feeds were studied. Six hours of additional cell growth from unlabeled substrates resulted in an OD<sub>600nm</sub> of about 13 (Figure 1).

The effects of enhancing cell density on the expression level were analyzed by SDS-PAGE. The specific protein production rate was kept constant over the time tested, and the generation of higher biomass leads to more recombinant protein yields.

The four deuterated HCDF set-ups for production of DCN-OmpG were programmed with a biomass accumulating feed that lasted 6 hours (Figure 2). After a prolonged lag phase the cells grew at

an easy rate, finally attaining ODs comparable to the nondeuterated experiments. The automatic switch from batch to fed-batch was initiated by the rising dissolved oxygen (DO) once the substrates had depleted.

The signal triggered the start of DO-based feeding (deuterated medium with unlabeled N- and C-sources) for further biomass enrichment. The DO allowed adjustment to the requirements of the cells. After six hours the feed automatically switched to labeled feed. The feeding rate was kept constant for sufficient protein production. After one hour the IPTG as inducer for protein production was automatically added. The duration of expression was another six hours, after which the feeding was stopped by the process control. The reactors were cooled for later cell harvest.

Table 2 shows the impact of the HCDF, as the specific protein production rate was even better than that of the shaking culture, producing an 11-fold higher yield of protein from the same volume of culture.

Two different NMR-spectra are shown in Figure 3. Spectrum A reflects <sup>15</sup>N labeling as <sup>1</sup>H-<sup>15</sup>N correlations. The original deuterated nitrogens were protonated due to the purification procedure with water-containing buffers. Spectrum B shows missing <sup>1</sup>H-<sup>13</sup>C-proton correlations, as deuterated carbon atoms keep the deuteron bound, even in water. The spots to be seen in spectrum B originated from the natural abundant <sup>1</sup>H-<sup>13</sup>C-proton correlation of the nondeuterated detergent used for refolding.

These results show that the HCDF labeling protocol is a useful tool for <sup>2</sup>H<sup>13</sup>C<sup>15</sup>N-labeled protein production under the requirements of NMR spectroscopy.

## Conclusions

Due to the toxic effects of D<sub>2</sub>O, deuteration of proteins is a challenge. Data from the literature often underline a required adaptation of cells to deuterated conditions via stepwise enhancement of the D<sub>2</sub>O content in the medium. This is time consuming and expensive.

We inoculated 99% deuterated medium relatively thickly (starting at OD<sub>600nm</sub> 0.25–0.3) with cells collected from preculture grown on nondeuterated rich medium. That led to good and reproducible results for cell growth and protein production. Following this strategy the HCDF was initialized at OD<sub>600nm</sub> 0.5 growing with about one-half of the speed of the water-based preliminary experiments. Nevertheless the same yield of recombinant protein could be achieved.

The depletion phase and the head start of the labeled feed with respect to

the induction is essential to ensure high labeling of the protein of interest. The NMR-spectra demonstrate the successful labeling of OmpG, producing a product suitable for NMR-supported structural biology, while the protocol shows the reduction of costs down by 25%.

The HCDF was used for a relatively difficult application of isotopic labeling of recombinant proteins. The system is applicable to all variants of unlabeled expression as well.

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### Table 1. Programmed Fermentation Parameters

Temperature	37°C
Stirrer speed	500–1,000 rpm
DO control via stirring	30%
pH control with 2 M NaOH	7.0
Biomass accumulation phase/unlabelled feed	0–6 h – 1 mL/h
Depletion phase	0.5 h
Labeled feed	7 h – 1 mL/h
Expression phase/ IPTG feed–Induction start	6 h/1 h after labeled feed started

### Table 2. Results and Costs of HCDF with the DASGIP Bioreactor System Compared to a Corresponding Shaking Culture Normalized to a Volume of 1 L

	Shaking culture	Factor	HCDF
Volume (mL)	1,000	1	1,000
Bio wet mass (g)	2.5	8.5	21.3
Amount of protein as solubilized inclusion bodies (mg)	30	11	330
Costs (in U.S.\$)*	840	2.75	2,310
\$ per mg solubilized inclusion bodies*	28	0.25	7

\*based on prices from 2006