

High Performance Cell-Free Wheat Germ Protein Expression System

Application Note

MEERCAT: Enabling large-scale quantitative proteome analysis

Abstract

Protein quantification by SRM (Selected Reaction Monitoring) analysis of peptides is the gold standard for quantitative proteomics in research and clinical applications. While initially focusing on few selected proteins, recent technologies allow for proteome-wide studies using large sets of isotope-labeled reference peptides. Instead of chemically synthesizing each peptide, multiple peptides can be concatenated into a “QconCAT”. After expression of the QconCAT gene, individual peptides are then released from the QconCAT in a co-digestion step with the analyte. MEERCAT now extends the QconCAT concept by shifting to CFS’ wheat germ cell-free expression system which enables highly parallel preparation of tens of thousands of standard peptides for studies on complex proteomes.

Introduction

Accurate protein quantification is important for understanding protein complexes, biological processes or for diagnoses from clinical samples [1]. Targeted mass spectrometry (MS) is the most universal and accurate approach, and different strategies have been developed to determine relative and absolute protein amounts e.g. by label-free methods, comparing samples by SILAC, or using an isotope-labeled reference protein (PSAQ) or peptide (AQUA). Stable-isotope-labeled reference peptides are preferred for proteomics as such reference peptides can be made to distinguish many different proteins in the same sample [2]. However, prior information on each peptide is needed before it can be used in MS experiments. Since peptide detectability is not fully predictable [3], each peptide must be experimentally verified and characterized. Reference databases like PeptideAtlas [4], ProteomicsDB [5], or ProteomeTools [6] provide information on thousands of peptides to help selecting suitable reference peptides for each

experiment. These peptides are commonly obtained by chemical synthesis, which is costly when many labeled peptides are required. Therefore, Beynon et al. developed **QconCATs** (Quantification **Concatamers**) to make the use of many peptides affordable [7, 8]. A QconCAT is an artificial protein made by gene design to encode multiple reference peptides that are released during co-digestion with analyte proteins. The known amount of the isotopically-labeled QconCAT allows for accurate, multiplexed preparation of several reference peptide standards for quantification of their unlabeled counterparts within a sample preparation. QconCATs have routinely been made in *E. coli* [8]. More recently, a shift to expression *in vitro*, using our wheat germ cell-free protein expression system, overcame several limitations of the *E. coli* system and has facilitated highly-multiplexed expression reactions for the simultaneous production of tens of thousands of reference peptides. This new cell-free method was termed “MEERCAT” for **Multiplexed Efficient Expression of Recombinant QconCATs** [9].

Application Note 4/2018: MEERCAT - scaling up QconCAT production

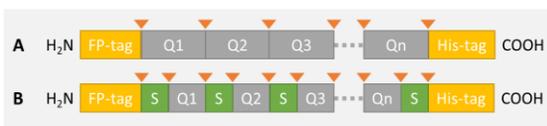


Figure 1: Schematic description of QconCAT proteins: In a QconCAT individual peptides (Q1 to Qn) are concatenated in one longer protein. The peptide sequences may be linked directly (A) or, more commonly, are separated by flanking sequences (S) that mimic digestion sites (orange triangles) in the native proteins (B). QconCATs have a His-tag at the C-terminus to assure the purification of the full-length protein. In MEERCAT an additional “mass-coded” FP-tag had been added to the N-terminus to barcode each QconCAT in multiplexed expression reactions.

Use of cell-free protein expression in MS

The wheat germ cell-free protein expression system has a long record of use in protein research, including the preparation of reference proteins for MS [10]. The universal power of our system for large-scale protein production was demonstrated by expressing over 18,000 human recombinant proteins from the AIST and ORFeome clone collections [11]. Those proteins were digested and then chemically labeled with mTRAQ Δ 4 to prepare a genome-wide peptide resource. The resulting database comprises 216,476 unique peptides for 17,973 proteins, representing about 86.3% of all (20,819) human protein-coding genes. However, chemical labeling can be fully avoided by direct incorporation of $^{13}\text{C}/^{15}\text{N}$ labeled lysine and arginine during a cell-free protein expression reaction [12]. Direct protein labeling in our wheat germ system was demonstrated in several studies e.g. working on transmembrane proteins [13] or using the FLEXIQuant method to prepare full-length reference proteins for MS experiments [14, 15]. Takemori et al. were the first to use this approach for high-throughput production of a stable-isotope-labeled library comprising 162 QconCATs covering 2201 selected peptides achieving a 25-fold gain in efficiency over previous QconCAT experiments [16]. In their protein expression and labeling experiments, they could achieve up to 99% labeling efficiency for incorporating [^{13}C , ^{15}N]-L-Lys and [^{13}C , ^{15}N]-L-Arg. This greatly increases the sensitivity of MS analysis and provides for a wider dynamic range than commonly

possible when using *in vivo* labeling methods. Thus, the wheat germ cell-free protein expression system is a preferable method to prepare stable-isotope-labeled reference proteins.

MEERCAT: Enhancing QconCAT Production

Following up on their first publication, the Takemori group at Ehime University and the Beynon group at the University of Liverpool [9] tested together whether the wheat germ cell-free system could overcome problems experienced while preparing QconCATs from *E. coli* cells. Based on the experience from working on over 100 individual QconCATs, about 1 out of 10 QconCATs could not be expressed at high levels in *E. coli*. Moreover, some of the QconCATs that were expressed in *E. coli* were proteolyzed during expression and subsequent purification, reducing their usefulness.

New expression vectors for the wheat germ system were prepared by PCR cloning starting from existing expression vectors to prepare a set of 12 QconCATs that could not be obtained from *E. coli* (11 cases failed to express, and one had been degraded). Therefore, this study kept on using inserts codon-optimized for expression in *E. coli*. Using the new expression vectors in small-scale 240 μl cell-free expression reactions, the team could obtain all QconCATs at an average concentration of about 0.1 $\mu\text{g}/\mu\text{l}$ with an incorporation rate for the isotope-labeled lysine and arginine of 99.6%, even though the templates had not been optimized for the wheat germ system. For one QconCAT, they could further show that the proteins made in the wheat germ system are less likely to be degraded as compared to working in *E. coli*. Obtaining some 20 to 30 μg for each QconCAT in a simple bilayer reaction enables hundreds of SRM assays, making this reaction scale suitable for routinely preparing reference proteins for use in MS studies within a day.

The open nature of a cell-free protein expression reaction offers great flexibility over the reaction conditions. This includes the use of several expression

Application Note 4/2018: MEERCAT - scaling up QconCAT production

Conclusion

The MEERCAT method demonstrates the benefits of the wheat germ cell-free protein expression system in the preparation of isotope-labeled standards for MS experiments. With the Premium PLUS Expression Kit for MS (CFS-PLUS-MS), CFS offers ready-to-use premixed reagents to perform protein labeling reactions in a simple three-step process. ¹³C and ¹⁵N-labeled lysine and arginine are already added to the reagents, and reaction conditions have been optimized to achieve about 99% labeling efficiency. The Premium PLUS Expression Kit for MS can be directly used to prepare QconCATs on a 226 µl scale as described in the MEERCAT publication.

Using cell-free protein expression can make it easier to obtain more QconCAT standards for protein MS or other applications like RePLiCAL, QconCAT proteins used for retention time standardization in proteomics [18]. We hope that the MEERCAT approach and individual QconCATs will pave the way to extend the scope of protein quantification experiments.

References

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Acknowledgement

We are very grateful for the support of Prof Robert J. Beynon at the University of Liverpool and Dr Nobuaki Takemori at Ehime University.

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