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Application Note

FLEXITau – Establishing a FLEXIQuant assay to quantify post-translational modifications of the human tau protein

Abstract

Post-translational modifications (PTMs) play an essential role for protein function, and thus are often related to medical conditions. One such example is the hyperphosphorylation of tau, a microtubule-associated protein, mostly expressed in neurons. Aggregation of tau relates to several neurodegenerative disorders, where the complex modification of tau may indicate the onset of such diseases. Therefore, a FLEXIQuant-based assay was developed to quantitatively studying PTMs of the tau protein *in vitro* and human disease. The FLEXIQuant PLUS Expression Kit from CellFree Sciences can be used to prepare stable-isotope-labeled reference proteins for use in mass spectrometry (MS) such as the analysis of tau described here.

Introduction

The FLEXIQuant (**F**ull-**L**ength **Ex**pressed Stable **I**sotope-Labeled Proteins for **Quant**ification) method developed by the groups of Drs. Judith and Hanno Steen in the Departments of Neurology and Pathology at Boston Children's Hospital (Boston, MA, USA) allows for in-depth quantitative characterization of proteins and their PTMs [1-3]. FLEXIQuant uses a wheat germ cell-free protein expression system [4] to prepare heavy, stable-isotope-labeled full-length reference protein standards. The isotope-labeled full-length protein standards are expressed as fusion proteins with a His affinity tag and an artificial peptide tag ("FLEX-peptide") at their N-terminus. During trypsinization, the FLEX-peptide is released from the full-length protein and can later be detected as a single peptide peak during MS analysis of the fragments. The FLEX-peptide is specifically labeled along with the rest of the protein during the *in vitro* expression reaction. Hence, the FLEX-peptide can be quantified in MS experiments by reference to an added, unlabeled, synthetic FLEX-peptide standard. In this way, the reference protein standard is indirectly quantified through the synthetic FLEX-peptide. This indirect quantification method enables working with only tiny amounts of labeled reference protein. In addition, the reference protein does not need to be extensively purified and biochemically quantified before use, making this an easy and effective way to prepare protein standards for MS assay development and later use in biochemical studies. It is important to note, that proteins made in certain wheat germ cell-free protein expression systems are commonly having no PTMs, because the necessary substrates have largely been removed during extract preparation. Therefore, all the peptides derived from full-length proteins made in these systems should have a defined mass that can be determined from the peptide sequence. PTMs can later be identified, and quantified, by reference to the unmodified proteolytic peptides derived from the isotope labeled reference protein. Because multiple peptides are generally obtained from a full-length

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protein standard, the method often allows to monitor PTMs at various amino acids along major parts of the protein of interest. This possibility had driven the development of a new assay in the Steen lab, denoted as "FLEXITau", to quantitatively studying post-translational modifications of the human tau protein [5].

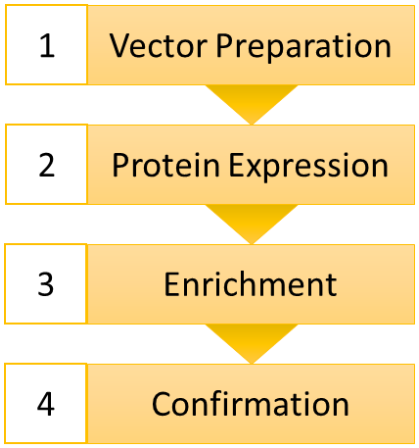

Figure 1: Preparation of a labeled reference protein requires four basic steps: 1. Preparation of an expression vector for protein of interest; 2. Protein expression and labeling in wheat germ cell-free protein expression system; 3. Enrichment of the reference protein, e.g. by use of His-tag; 4. Confirmation of protein expression.

Materials and methods

Isotope-labeled tau was prepared using an amino-acid-free wheat germ extract of the WEPRO8240H series (CellFree Sciences, Japan). The amino-acid-free extract provides more flexibility to select different amino acids for labeling with about 97% incorporation efficiency. In the case of FLEXITau, a triple labeling strategy [lysine K8 ($^{13}C_6$ $^{15}N_2$), arginine R10 ($^{13}C_6$ $^{15}N_4$), aspartate D5 ($^{13}C_4$ $^{15}N_1$)]. Moreover, the WEPRO8240H wheat germ extract was precleared on a Ni resin to reduce the number of background proteins when using a His-tag for enriching the labeled reference protein in an affinity purification step (*Figure 1*). Before use, tau reference protein preparations were incubated with lambda protein phosphatase for 30 min to remove possible phosphate groups and to ensure working with unmodified peptides. Further details on conducting FLEXITau experiments can be found in the supporting information to [5].

Results and discussion

Studying PTMs of tau in clinical samples is complex because of multiple modifications at different sites varying depending on the biological context. Specific antibodies directed against a phosphorylated serine and threonine are often used, but they do not provide information on the total number of phosphorylations and other modifications. Protein MS is a powerful approach to study PTMs, nonetheless the limited accuracy of low-resolution MALDI-TOF analysis had provided inconsistent data on the phosphorylation status of the tau protein [6]. This motivated the development of FLEXITau, where an added full-length, stable isotope-labeled tau standard allowed for sensitive and reproducible data acquisition using an SRM (selective reaction monitoring) workflow.

FLEXITau was developed based on the FLEXIQuant method that had already been used for example to analyze mitotic regulators such as CDC27 [2] or KifC1 [7] in whole cell lysates. To study the extend of protein modifications, a quantitative workflow is required, where a reference standard comprising the entire protein is preferred over other approaches using few selected reference peptides. The wheat germ cell-free protein expression system offers effective means for the preparation of full-length reference proteins, where the open reaction format enables easy protein labeling reactions. Over 18,000 human recombinant proteins have been prepared in this way to obtain reference peptides for targeted proteomics [8]. The tau protein within the sarkosyl insoluble fraction of brain lysates from tauopathy patients can be directly detected by MS without enrichment from the sample. Since there is no enrichment step for the endogenous tau protein, it is necessary to enrich the reference tau standard after completion of expression reaction in the wheat germ system. Otherwise, the reference protein may be too much diluted with wheat proteins from the crude reaction mixture. The His-tag added along with the FLEX-peptide tag in the fusion protein can be used to easily and rapidly enrich the standard. The purification step for the reference protein is not required when the target protein must be enriched for detection. In such cases, the reference protein can

be enriched together with the target protein within the sample. As said, the proteins made in this wheat germ system are commonly lacking modifications, but to ensure working with an entirely dephosphorylated standard the labeled tau reference protein is further incubated with lambda protein phosphatase to remove any possible phosphate groups. The sample ("light protein") and reference ("heavy protein") are mixed early in samples processing to minimize quantification errors caused by samples losses or technical problems. All further steps during sample preparation are applied to light and heavy proteins together and follow standard processes for protein MS experiments. Only the light FLEX-peptide reference must be added before the mass spectra can be acquired. This FLEX-peptide is needed to quantify the heavy peptides derived from the tau reference standard.

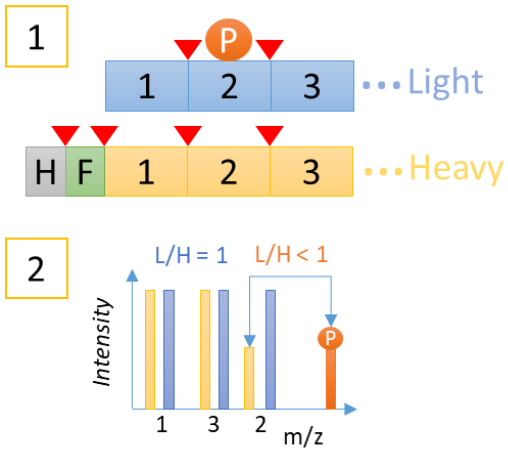

Figure 2: Detecting modified peptides: 1. The "light" protein in the sample is mixed with the "heavy" protein standard. Both proteins are processed together where peptides are released after tryptic digestion (red triangles); 2. Peptides derived from both proteins are then detected by MS. Whereas the ratio of light to heavy remains unchanged for unmodified peptides (here peptide 1 and 3), the ratio of light to heavy is changed once a phosphorylated peptide is observed (here peptide 2). If only one modification occurs per peptide, the amount of modified peptide can be determined by reference to the ratio of light to heavy; additional considerations are required if multiple modifications occur on the same peptide [5]. *H = His-tag, F = FLEX-peptide*

PTM detection is done by comparing peptide pairs of light and heavy isotopologues (*Figure 2*). The mixing ratio between the tau protein in the sample and the tau reference standard is calculated for unmodified peptide pairs. Knowing this ratio, modified peptides can be identified where the amount of unmodified peptide within the sample is reduced in favor of the modified form. In the best case, the amount of modification can be determined from the change to the mixing ratio (one modification per peptide); refer to the publication for more details on the analysis of peptides with multiple modifications [5]. Following this approach, the FLEXITau assay can reproducibly achieve a sequence coverage of up to 75% using 23 peptides. For a concentration range of 0.4 to 400 fmol a R^2 value of >0.98 was observed for approximately 90% of the peptides [5]. FLEXITau was successfully used to get a better overview on the PTM landscape of tau in AD post-mortem brain samples and offers great promises for future use in clinical studies.

Conclusion

The FLEXITau assay demonstrates the benefits of working with a full-length reference protein in MS studies. The FLEX-peptide within the reference protein standard enables not only quantitative SRM experiments but was used here to obtain additional information on the PTM status of the tau protein.

It is easy to establish FLEXIQuant experiments by using the new *FLEXIQuant PLUS Expression Kit from CellFree Sciences that provides ready to use reagents for preparing labeled proteins starting directly from a DNA template. A premixed reaction mixture dedicated for MS experiments (also available separately in the Premium PLUS Expression Kit for MS) is used for incorporation of $^{13}C/^{15}N$ labeled lysine and arginine. High incorporation rates of about 99% for the labeled $^{13}C/^{15}N$ labeled lysine and arginine can be achieved because of the use of a wheat germ cell-free expression system optimized for protein labeling. The kit further provides a positive control, an expression vector to prepare a template for fusion protein expression together with the His-tag and FLEX-peptide, and the FLEX-peptide reference peptide. The protein expression and labeling experiment is highly reproducible to repeatedly prepare labeled reference proteins for protein MS. We hope that the new FLEXIQuant PLUS Expression Kit will help to develop more assays for protein MS

and proteomics studies like the FLEXITau assays described here.

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Acknowledgement

*The FLEXIQuant Protein Expression Kit was developed in collaboration with Hanno Steen, PhD, from the Department of Pathology at Boston Children's Hospital based on the original publication:

FLEXIQuant: a novel tool for the absolute quantification of proteins, and the simultaneous identification and quantification of potentially modified peptides. Singh S, Springer M, Steen J, Kirschner MW, Steen H.: J Proteome Res. 2009;8:2201-2210.

We are very grateful for the support of Dr. Hanno Steen.

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