Full-Length Expressed Stable Isotope-Labeled Proteins for Quantification

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FLEXIQuant, short for Full-Length Expressed Stable Isotope-Labeled Proteins for Quantification, utilizes stable isotope-labeled (“heavy”) full-length recombinant proteins which are expressed in vitro using WEPRO® and other reagents from CellFree Sciences (CFS). The detailed workflow of FLEXIQuant is depicted in Figure 1, with more details published elsewhere.1,2 The isotopic labeling of the heavy protein standards is achieved by incorporating 15N- and/or 13C-labeled amino acids, preferably lysine and arginine. Prior to trypsinization, similar amounts of the heavy protein standard and the unlabeled endogenous sample protein are combined. The unlabeled (“light”) protein of interest and the heavy protein standard can be purified separately beforehand. Alternatively, the WEPRO® extract with the recombinant heavy protein standard can be mixed with the cell or tissue lysate containing the light endogenous protein of interest prior to simultaneously purifying by immunoprecipitation the light and the heavy proteins. After trypsinization, the digest is analyzed by mass spectrometry (Figure 1 and 2). All unmodified peptides will be present as pairs featuring the light and the heavy isotopeologue, whereby the light-to-heavy (L/H) intensity ratio of these peak pairs reflects the initial mixing ratio. Any deviation from this ratio indicates a) that a fraction of the endogenous peptides was modified and thus shifted in the mass-to-charge (m/z)ratio and the retention time domain, and b) the extent of this modification: if the endogenous peptide is present only at 20% of the intensity expected based on the ratio of all other modified peptides, it can be assumed that 80% of this peptide was modified.

An additional feature of the FLEXIQuant approach is the presence of a unique peptide, the FLEX peptide, next to the His6-tag used for purification. This FLEX peptide can be used to determine the absolute quantification of the heavy recombinant protein using “inverse” isotope dilution mass spectrometry – “inverse” as the synthetic peptide used for quantification is not labeled because the analyte of interest, i.e. the full-length recombinant protein, is labeled. Once the absolute concentration of the heavy recombinant protein is determined, the absolute concentration of the endogenous light protein can be inferred.

An example application of FLEXIQuant is shown in Figure 2. In this particular example, HeLa S3 cells were arrested in prometaphase using nocodazole.1 Aliquots were removed four, eight and 12 hours after initiating the nocodazole treatment. After cell lysis in the presence of phosphatase inhibitors, wheat germ extract containing heavy CDC27 was added to the lysate. After cell lysis, the digest was analyzed by liquid chromatography mass spectrometry (LC/MS). All observable CDC27-derived peptides presented themselves as isotopeologue pairs. Those peptides that became modified during the incubation with nocodazole showed a decrease in the L/H ratio (see Figure 2A and B), which i) identifies the modified region of CDC27 during mitotic arrest, ii) provides information about the extent of modification, and iii) provides insights into the modification kinetics. More detailed analysis identifies the modifications as phosphorylation (see Figure 2A, green arrow).

Additional examples for applications of the FLEXIQuant strategy and derivatives thereof, such as FLEXIQinase,5 (Figure 1) have been published.3,4,5

(continued)
**Synthesis of a heavy isotope-labeled protein standard**

1. Clone gene into WGE/eukaryotic expression vector containing the FLEX-tag
2. Standard plasmid prep, phenol-chloroform cleanup (1mg/ml final concentration)
3. Transcription reaction (20 µL)
4. Translation reaction under supply of (isotope labeled) amino acids (200 µL)
5. Ni affinity purification of heavy standard (optional for classical FLEXIQuant)

**Classical FLEXIQuant analysis**
- Mix heavy standard with cell lysate of any biological sample
- Optional: Immunoprecipitation of endogenous protein/synthesized standard
- Separation by SDS-PAGE and in-gel tryptic digestion, also producing the FLEX peptide
- Add known quantity of light FLEX peptide
- Mass spectrometric analysis

**FLEXIQinase analysis**
- In vitro kinase assay using light and heavy substrate
- Phosphorylation by Kinase 1
- Remove
- Phosphorylation by Kinase 2
- Mix
- Separation by SDS-PAGE and in-gel tryptic digestion
- Mass spectrometric analysis

**Absolute quantification of heavy standard and in turn endogenous protein**

**Relative quantification of light and heavy peaks to extract biological information**

**Peak chase profiling of all possible P-sites and extraction of kinase mechanism**

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**Figure 1.** FLEXIQnase workflow and its applications. Top: Common to all FLEXIQuant analyses is the *in vitro* expression of a heavy protein standard. The gene of interest is cloned into the wheat germ expression vector, which is then separately transcribed and subsequently translated in wheat germ extract (CellFree Sciences, www.cfsciences.com) under supply of heavy isotope-labeled amino acids. Purification of the heavy standard can be achieved by Ni affinity purification via the His6-tag. Left: In the classical FLEXIQuant approach, the wheat germ-expressed heavy protein is mixed with any type of biological sample, such as HeLa cell lysate. For enrichment, heavy and light proteins can be co-purified by immunoprecipitation. The protein mix is separated by SDS-PAGE and gel bands of interest are in-gel trypsinized. A known amount of (light) FLEX peptide is added and the peptide mix is analyzed by mass spectrometry. Absolute quantity of the light protein and a relative peptide quantification can then be determined by peak integration of corresponding light and heavy signals, revealing modified regions of the protein in the endogenous sample. Right: For the FLEXIQnase approach, the substrate of interest is differentially expressed in its heavy and light version. *An in vitro* two-kinase assay is performed, yellow circles indicating phosphorylation by Kinase 1, orange circles by Kinase 2. In-gel trypsinization is followed by mass spectrometric analysis and peak chase profiling of all possible phosphorylation states is performed in order to extract kinase mechanism.
Figure 2: FLEXIQuant analysis of CDC27 to unravel phosphorylation dynamics during mitosis. Hela cells were arrested in prometaphase using nocodazole. After defined time periods, aliquots were removed, lysed and mixed with a heavy full-length version of CDC27 expressed with a Premium Plus Expression Kit for MS manufactured by CFS. Upon immunoprecipitation of the light and heavy CDC27, the sample was trypsinized and analyzed by LC/MS. A) m/z-region of the light and heavy unmodified (red oval) version of the peptide QPETVLTETPQDTIELNR and the singly phosphorylated version (green arrow). B) FLEXIQuant analysis (L/H ratio vs. peptide from N- to C-terminal) of all observed CDC27-derived peptides clearly depicted the extent of modification in the N-terminal half of CDC27 (indicated in C) that increased with prolonged duration of nocodazole treatment.

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References of the original FLEXIQuant paper, a detailed protocol and three different applications of FLEXIQuant

Original paper:

Detailed protocol:

Three different applications: