



CIL

Cambridge Isotope Laboratories, Inc.
isotope.com **Eur isotop**
A CAMBRIDGE ISOTOPE LABORATORIES COMPANY

APPLICATION NOTE 40

Full-Length Expressed Stable Isotope-Labeled Proteins for Quantification

Waltraud Mair,¹ Sasha Singh,^{1,2,3} Judith Steen¹ and Hanno Steen²

1. F.M. Kirby Neurobiology Center, Boston Children's Hospital, Boston, MA USA

2. Department of Pathology, Boston Children's Hospital, Boston, MA USA

3. Center for Interdisciplinary Cardiovascular Sciences,
Brigham and Women's Hospital, Boston, MA USA

FLEXIQuant, short for **F**ull-**L**ength **E**xpressed **S**table **I**sotope-**L**abeled **P**roteins for **Q**uantification, 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 isotopologue, 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. The light endogenous and the heavy exogenous CDC27 were co-purified by immunoprecipitation. After trypsinization, the digest was analyzed by liquid chromatography mass spectrometry (LC-MS). All observable CDC27-derived peptides presented themselves as isotopologue 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)

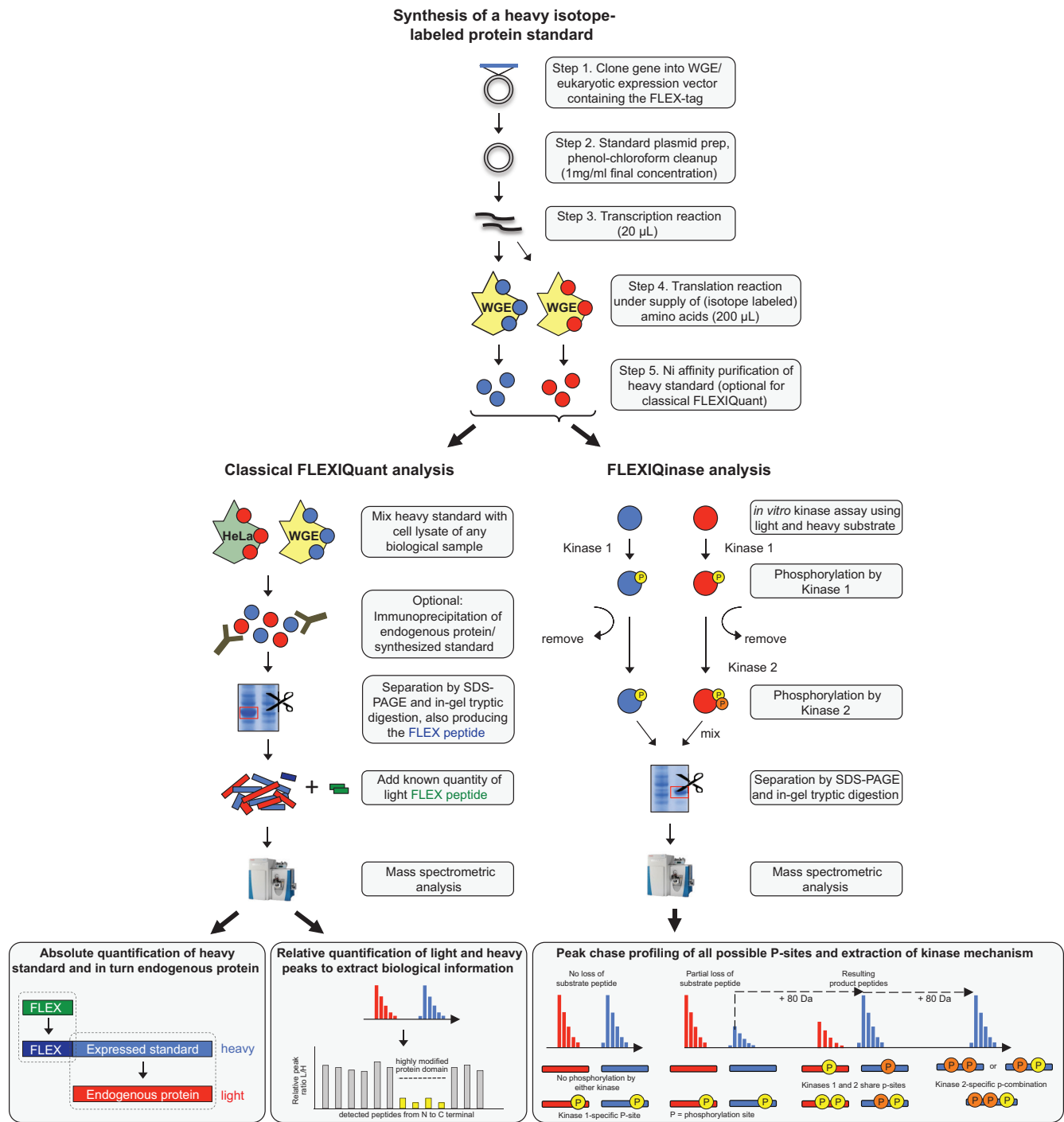


Figure 1. FLEXIQuant workflow and its applications. **Top:** Common to all FLEXIQuant analyses is the *in vitro* expression of a heavy protein standard. The gene of interest is cloned into the wheat germ expression vector, which is then separately transcribed and subsequently translated in wheat germ 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 FLEXIQinase 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.

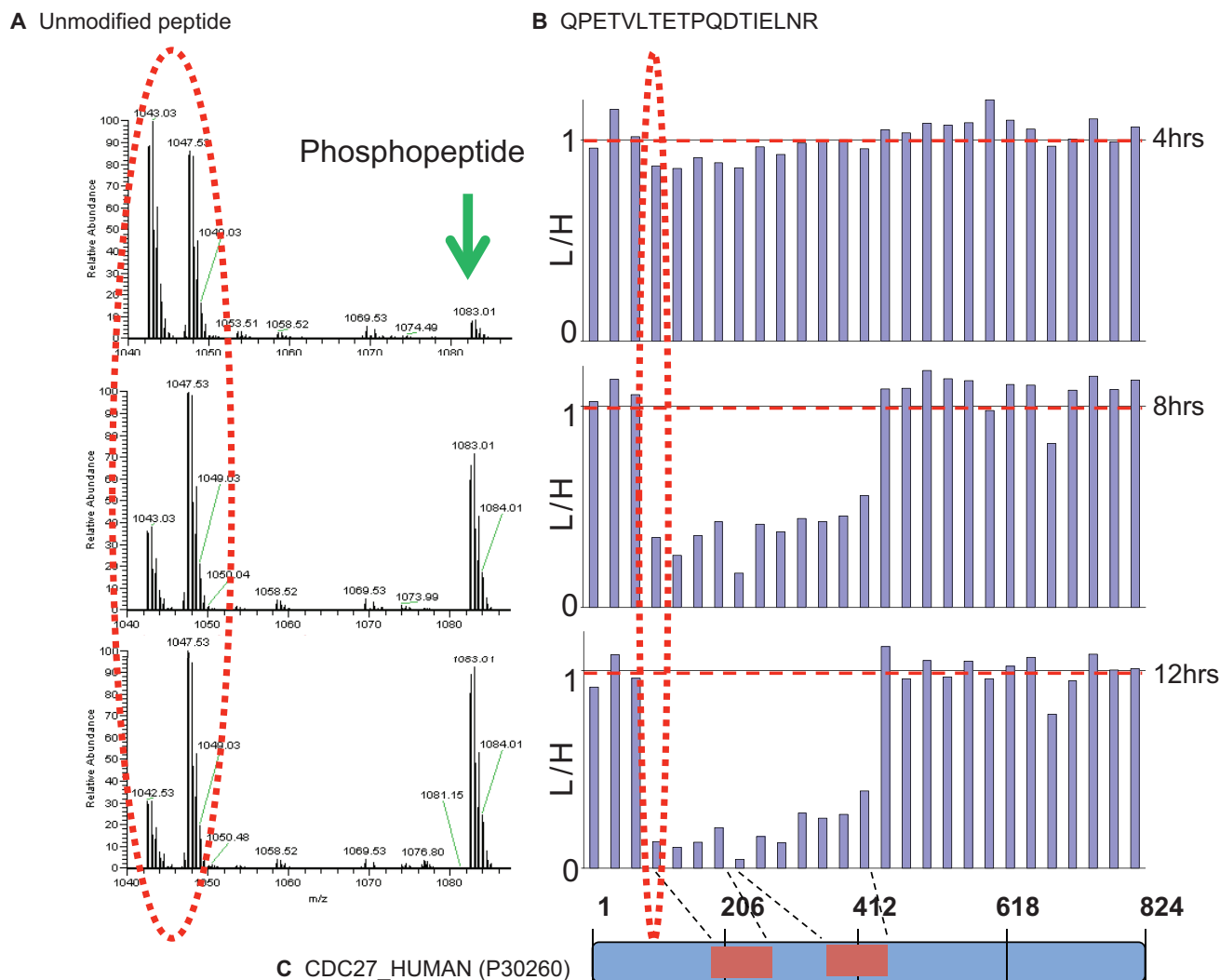


Figure 2. FLEXIQuant analysis of CDC27 to unravel phosphorylation dynamics during mitosis. HeLa cells were arrested in prometaphase using nocodazole. After defined time periods, aliquots were removed, lysed, and mixed with a heavy full-length version of CDC27 expressed with a Premium Plus Expression 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.

Related Products

Catalog No.	Description
CFS-FLEX-MS*	FLEXIQuant Protein Expression Kit Contains all required transcription and translation agents, including $^{13}\text{C}/^{15}\text{N}$ -labeled Lys and Arg, EU-E01-TEV-N1-FLEX-tag vector and FLEX peptide. Kit can perform eight 226 μL reactions with a positive control.
CFS-EDX-PLUS-MS*	Premium PLUS Expression Kit for MS Protein synthesis kit with $^{13}\text{C}/^{15}\text{N}$ -labeled lysine and arginine for MRM/SRM mass spectrometry

Catalog No.	Description
CFS-EDX-PLUS*	Premium PLUS Expression Kit Contains all required transcription and translation reagents. Reaction scale is 226 μL . Kit contains expression vector, positive control and split-PCR primers
CNLM-539-H	L-Arginine-HCl ($^{13}\text{C}_6$, 99%; $^{15}\text{N}_4$, 99%) Free amino acid at 98% chemical purity
CNLM-291-H	L-Lysine-2HCl ($^{13}\text{C}_6$, 99%; $^{15}\text{N}_2$, 99%) Free amino acid at 98% chemical purity

*Manufactured by CellFree Sciences and distributed by Cambridge Isotope Laboratories, Inc. For more information regarding products from CellFree Sciences, please visit CFSciences.com.

For more information, visit isotope.com → Applications → Biomolecular NMR → *in situ* Protein Expression

(continued)

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

Original paper:

1. Singh, S.; Springer, M.; Steen, J.; Kirschner, M.W.; Steen, H. **2009**. FLEXIQuant: a novel tool for the absolute quantification of proteins, and the simultaneous identification and quantification of potentially modified peptides. *J Proteome Res*, 8(5), 2201-2210.

Detailed protocol:

2. Singh, S.; Kirchner, M.; Steen, J.A.; Steen, H. **2012**. A practical guide to the FLEXIQuant method. *Methods Mol Biol*, 893, 295-319.

Three different applications:

3. Singh, S.A.; Winter, D.; Kirchner, M.; Chauhan, R.; Ahmed, S.; Ozlu, N.; Tzur, A.; Steen, J.A.; Steen, H. **2014**. Co-regulation proteomics reveals substrates and mechanisms of APC/C-dependent degradation. *EMBO J*, 33(4), 385-399.
4. Zhang, X.; Abreu, J.G.; Yokota, C.; MacDonald, B.T.; Singh, S.; Coburn, K.L.; Cheong, S.M.; Zhang, M.M.; Ye, Q.Z.; Hang, H.C.; Steen, H.; He, X. **2012**. Tiki1 is required for head formation via Wnt cleavage-oxidation and inactivation. *Cell*, 149(7), 1565-1577.
5. Singh, S.A.; Winter, D.; Bilimoria, P.M.; Bonni, A.; Steen, H.; Steen, J.A. **2012**. FLEXIQinase, a mass spectrometry-based assay, to unveil multikinase mechanisms. *Nat Methods*, 9(5), 504-508.

