Technical note

# MilQuant: A free, generic software tool for isobaric tagging-based quantitation

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## ARTICLE INFO

## ABSTRACT

Isobaric tagging techniques such as iTRAQ and TMT are widely used in quantitative proteomics and especially useful for samples that demand *in vitro* labeling. Due to diversity in choices of MS acquisition approaches, identification algorithms, and relative abundance deduction strategies, researchers are faced with a plethora of possibilities when it comes to data analysis. However, the lack of generic and flexible software tool often makes it cumbersome for researchers to perform the analysis entirely as desired. In this paper, we present MilQuant, mzXML-based isobaric labeling quantitator, a pipeline of freely available programs that supports native acquisition files produced by all mass spectrometer types and collection approaches currently used in isobaric tagging based MS data collection. Moreover, aside from effective normalization and abundance ratio deduction algorithms, MilQuant exports various intermediate results along each step of the pipeline, making it easy for researchers to customize the analysis. The functionality of MilQuant was demonstrated by four distinct datasets from different laboratories. The compatibility and extendibility of MilQuant makes it a generic and flexible tool that can serve as a full solution to data analysis of isobaric tagging-based quantitation.

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# 1. Introduction

Advances in instrumentation and techniques over the past decade have helped proteomics mature into the stage of quantitative profiling of proteomes. A variety of quantitation approaches have been developed and applied in an increasing number of research fields [1], among which stable isotope-based techniques have been especially favored over gel-based quantitation due to its high throughput, sensitivity, and reproducibility. Researches conducted in cell cultures have benefited greatly from metabolic labeling approaches such as stable isotope labeling by amino acids in cell culture (SILAC) [2], whereas *in vitro* labeling [3], also applicable in cell culture studies, is required for specific sample types, especially clinical samples such as serum, saliva, and cerebrospinal fluid. Moreover, while most stable isotope-based quantitation approaches are capable of simultaneous comparison of up to three samples, isobaric tags for relative and absolute quantitation (iTRAQ) [4] and tandem mass tags (TMT) [5], two commercially available isobaric tagging techniques, offer higher multiplexing capacity of up to eight multiplex channels. Altogether, these advantages made isobaric tagging based techniques popular choices for relative proteome quantitation.

With isobaric tagging techniques, relative quantities of peptides become apparent when the parts of tagging reagents fall off from these precursor peptide ions, producing reporter ions that appear in the tandem MS spectra at multiplexed m/z and with varying intensities, which serves as the basis for the deduction of relative protein abundances. Traditionally, TOF has been the mass analyzer of choice for production and acquisition of reporter ions, while the usage of ion trap (IT) is limited by the "one-third rule" [6]. However, recent development of novel dissociation methods, including pulsed Q dissociation (PQD) [7], electron transfer dissociation (ETD) [8] and high energy collision dissociation (HCD) [9], has also enabled ion trap (IT) and Orbitrap for such tasks. As a result, the diversity in mass spectrometry (MS) collection approaches and native acquisition files formats is greatly increased. Added with the diversity in choices of database searching, intensity normalization, and relative abundance computation methods, this could result in a great many variations on the theme of data analysis. Among the available tools in this field, a lot of them were developed before the advent of novel dissociation approaches and thus not directly compatible, whereas most other software tools, such as Mascot and Proteome Discoverer, works in an integrated fashion that reports only the ultimate relative abundance results, making it difficult for researchers to tailor the entire analysis protocol to their needs and to compare results from different instrumentation platforms.

In this paper, we present MilQuant (mzXML-based isobaric labeling quantitator), an open source software that is compatible with all mainstream mass spectrometers, tandem MS dissociation approaches, and database search algorithms that have been reported in isobaric tagging-based quantitation researches. It also offers a panel of effective normalization and relative abundance combination methods. Furthermore, the analysis with MilQuant is highly flexible pipeline, with each step generating a handful of output files to facilitate researchers to customize their downstream handling. Finally, we demonstrate the merits of MilQuant with four datasets collected with different approaches.

# 2. Technical basics of MilQuant

Since the identification of peptide and protein is the prerequisite for their quantitation, a typical quantitation workflow for isobaric tagging data usually import peptide and protein identities and associate them with peptide relative abundances indicated by the intensities of reporter ion peaks extracted from tandem MS spectra. These intensities will then undergo a series of transformations and integration into the final forms of relative peptide and protein abundance ratios [3].

Specifically, the MilQuant pipeline consists of the six following steps (Fig. 1): (i) reporter ion peak extraction, (ii) importing and optional filtering of protein and peptide hits, (iii) mapping spectral ratios to corresponding peptide sequences, (iv) isotopic impurity correction, normalization for inequality in amounts of samples for labeling, (v) an optional VSN normalization, and (vi) combination into peptide and protein abundance ratios.

MilQuant was written in Python and R programming languages. The source code is freely available at http://
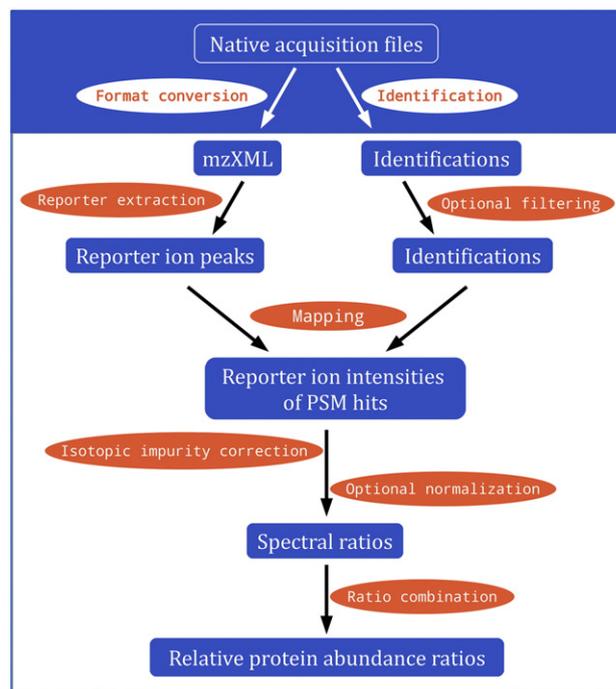


Fig. 1 – MilQuant's workflow. Preparations before MilQuant include conversion of native acquisition files and identification. Afterwards, MilQuant starts by extracting reporter ion peaks and mapping them to identified spectra and peptide sequences. Intensities of reporter ion peaks from qualified spectra are then corrected for isotopic impurities and normalized. Finally, peak intensities or their relative ratios of distinct spectra are combined into the relative abundance ratios of the corresponding peptides and proteins. PSM: peptide–spectrum match.

code.google.com/p/milquant with accompanying exemplary data, tutorial, and user manual. Detailed experimental procedures and results, along with figures of user interfaces, are described in the supplementary materials of this paper.

## 3. Features of MilQuant

### 3.1. Comprehensive native acquisition file format compatibility

While proprietary software programs are capable of performing quantitation, some of them can only process data collected by mass spectrometers from the same vendor. More importantly, the manner that quantitation is achieved is not easily customizable. First, quantitation is based on identification algorithms offered by the tools, which are not always the users' choices. Moreover, these tools mostly report only the final abundance ratios, while omitting the exportation of intermediate results such as peptide/protein identification before and after filtering, or reporter ion peak intensities before and after certain transformation, which could greatly facilitate users in making minor alterations to the protocol without implementing a new one from the beginning.

On the other hand, among a handful of public software in this field [3], despite their higher customizability, most could only process data produced by TOF mass spectrometers. Up to now a total of two types of mass spectrometers, four tandem MS dissociation methods, and two spectra collection modes (single or alternating types of tandem MS spectra for the same precursors) have been reported to be applicable for data collection, which leads to up to nine possible varieties in the data structure of native acquisition files, a complicated situation that could benefit from a generic solution.

MilQuant addresses the format diversity issue by means of utilizing generic MS data formats. Currently it directly supports mzXML [10] and mzData [11], two widely accepted formats that are compatible with most mainstream mass spectrometers and easily converted by freely available software such as ProteoWizard [12], Trans-Proteomic Pipeline (TPP) [13], and X2XML. MilQuant also is under development to directly read other generic MS data formats, including mzML [12] and PRIDE XML [6]. Starting with these formats, MilQuant is able to process various formats and structure of raw MS data, as demonstrated in the following sections (Fig. S1).

### 3.2. Highly flexible analysis protocol

Peptide and protein identifications serve as the basis of any quantitation workflow. MilQuant addresses only the quantitation aspect, leaving users with full freedom to use the identification protocol of their own choice. Currently MilQuant supports identification results from database search engines Mascot, X!Tandem, and Phenyx, as well as more sophisticated algorithms Proteome Discoverer, ProteinPilot, and TPP. For other identification programs, a compilation of all necessary input information in appropriate formats will also suffice the prerequisites for running MilQuant. MilQuant also offers an optional step of peptide and protein hits filtering for users who wish to experiment with different subsets of proteins and peptides (Fig. S2).

On the LTQ-Orbitrap series of mass spectrometers the acquisition scheme of pairs of MS/MS spectra from the same precursors was often adapted, typically with a CID spectrum
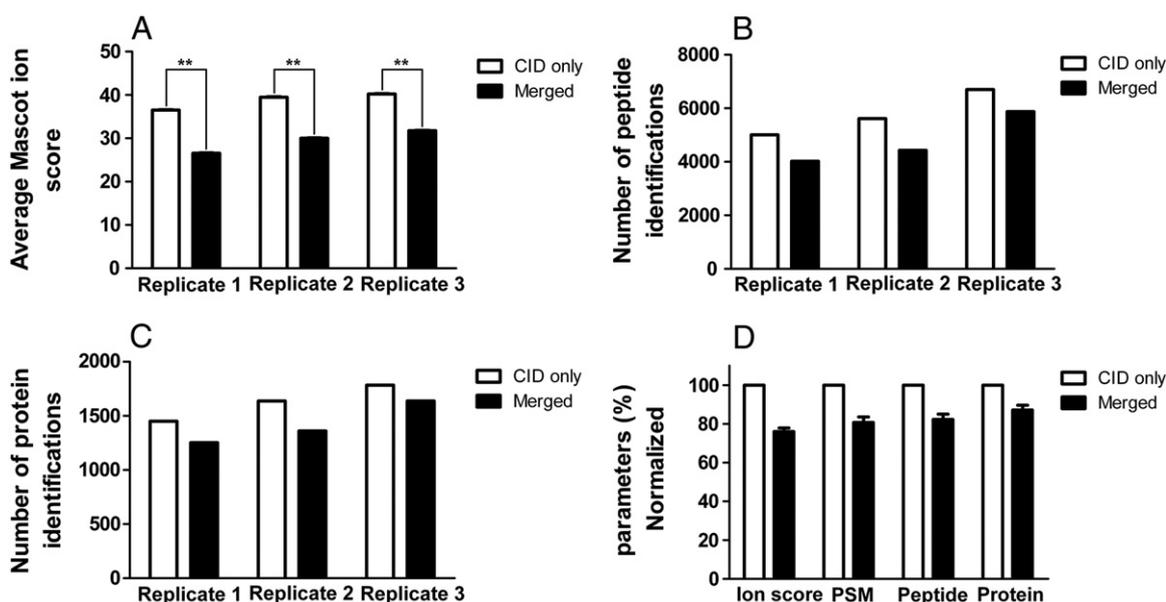


Fig. 2 – Identification results before and after merging raw reporter ion peaks into CID spectra from three replicate experiments. (A) Average Mascot ion scores, with an average 37082 spectra searched per replicate. (B) Number of peptide identifications at false discovery rates of 1%. (C) Number of protein identifications at false discovery rates of <1%. (D) Relative average Mascot ion scores and numbers of PSM, peptides and proteins identifications at false discovery rates of ≤1% after normalization against corresponding values before spectra merging, which were set as 100%. Error bars represents standard error of the mean. ** indicates $p < 0.01$, paired Student's t-test. PSM: peptide–spectrum match.

intended for identification, followed by an HCD spectrum for quantitation. In such cases the reporter ion peaks from the trailing spectra were usually inserted into the leading ones. However, this "merging" strategy has certain limitations. Peaks of reporter ions are usually much more intense than those in the original spectra, and this could result in an average reduction by 20% in numbers of peptide–spectrum matches as well as peptide and protein identifications when searched with the Mascot algorithm (Fig. 2, Supplementary methods). Normalizing reporter ion peak intensities to unity [9,14] avoids this identification loss, but causes permanent loss of original intensity information that is required for intensity-based abundance ratio computation, which has been shown to produce more accurate quantitation in certain datasets [15]. Here we propose a "mapping" strategy, which associates peptide–spectrum matches (PSMs) with the corresponding reporter ion peaks while leaving both parts intact (Fig. S3). By adapting this method, MilQuant offers the users the possibilities of both ratio-based and intensity-based relative abundance deduction.

MilQuant also seeks to provide as much convenience as possible for users who seek to apply computation algorithms other than those offered. In MilQuant each of the six steps generates a handful of results in spreadsheets. Using these intermediate results, users can modify, skip, or add extra pieces of data processing that would still fit into the jigsaw of quantitation workflow, as long as the input data conform to the boilerplate formats provided in the MilQuant software package.

### 3.3. Common, effective computation algorithms

Raw reporter ion intensities are usually corrected for isotopic impurity, normalized and combined into relative abundance ratios at the peptide/protein level. While the impurity correction is a straightforward process, normalization and ratio combination are fields that are open to exploration. A number of algorithms have been proposed and applied [16,17], among which the most frequently used ones are global normalization based on correction factors derived from the sum or median of all reporter ion peak intensities, as well as combing ratios by taking the median, arithmetic averages, or intensity-weighted averages.

MilQuant performs impurity correction as usual [18] and has three preset sets of correction parameters for iTRAQ 4-plex, iTRAQ 8-plex and TMT 6-plex, respectively (Fig. S4). Afterwards, MilQuant offers correct factor based normalization methods and an optional peak filtering. For spike-in experiments, an output file without such sample amount normalization is also exported. More advanced normalization is designed as optional steps in the MilQuant pipeline. Currently, MilQuant has incorporated an efficient transformation named variance stabilizing normalization (VSN), a robust solution for the issue of heterogeneity of variance among peaks of varying intensities [16,19]. It has also been adapted in IsobariQ [20], a software tool that uses identification results produced by the Mascot search engine for quantitation. In MilQuant VSN is presented as an optional step and can be performed alone, or before or after the global normalization. As MilQuant accepts results generated by any identification algorithm, the applicability of VSN is extended

beyond Mascot. In addition, since MilQuant the preserves reporter ion peaks intensities rather than normalize them to unity, VSN could produce more relevant results as this normalization method is intended to address the dependence of variance on peak intensities (Fig. S6). As always, users can perform their own processing and return to the MilQuant pipeline at the next step.

The journey of quantitation with MilQuant ends at ratio combination. Relative abundances at both the protein and the peptide levels bear biological relevance and are computed in MilQuant. Beside the common methods of taking median and arithmetic average of abundance ratios, the intensity-based combination mode is also available. On both spectra and peptide levels, abundance ratios can come from intensity-weighted averaging and from division of the intensity sums of different multiplex channel (Fig. S5).

### 3.4. Easy to handle and time-efficient

MilQuant provides user-friendly interfaces (Fig. S1–S5) as well as documents of tutorial and algorithm details. As reporter ion peak extraction can be performed before or in parallel with identification, the rate-limiting step occurs at spectra mapping. On our computer with 2.4 GHz CPU and 2 GB RAM, it typically takes about 30 minutes to process 10,000 spectra, while other steps finish almost instantaneously. The total running time, including mzXML conversion, identification, and full MilQuant quantitation, on our datasets of approximately 8000 MS/MS spectra per run, averaged at about 5 minutes per run, which was comparable with IsobariQ [20] and Proteome Discoverer (about three hours for 45 files).

## 4. Application of MilQuant

To demonstrate its validity and applicability, we applied MilQuant on four datasets both collected in-house and in other laboratories [16,21,22]. The native acquisition files come from different types of mass spectrometers (TOF and Orbitrap), acquisition schemes (CID in TOF, and HCD and CID-HCD for Orbitrap) and used different labeling reagents (iTRAQ 4-plex and iTRAQ 8-plex).

On our in-house CID-HCD dataset, peptides were obtained from an SDS-PAGE gel slice and labeled with iTRAQ 4-plex reagents (114:115:116:117 = 1:1:2:5). We used IsobariQ, Mascot, MilQuant and Proteome Discoverer and compared the performance of these four tools (Supplementary methods). As a measure of quantitation accuracy, we compared the proportions of proteins whose abundance ratios fell within the range of 30% relative error from the expected ratios, and found comparable performance among these tools (Fig. 3A, Table S1). Generally, less than 20% of all quantified proteins have aberrant ratios that deviated from the expected ratios by more than 30%.

We then used MilQuant on previous reported datasets collected by a Q-TOF mass spectrometer. Same amounts of samples were labeled with iTRAQ 4-plex reagents [16]. We also evaluated the distribution of relative errors of quantitation results derived by IsobariQ, Mascot, and MilQuant (Fig. 3B, Table S2). Consistent with the Orbitrap dataset, less than ten
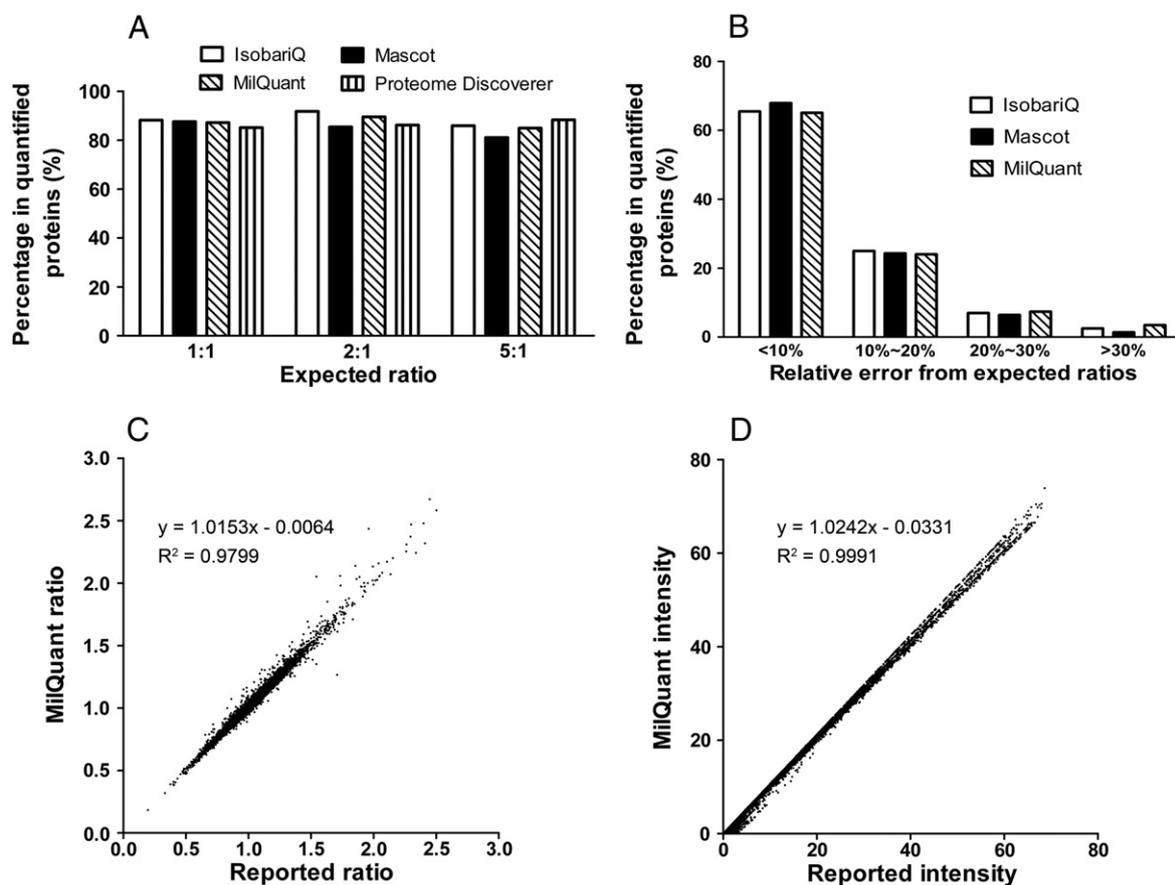
Fig. 3 – Demonstration of MilQuant's functionality on four distinctly different datasets. (A) Comparison of quantitation accuracy of IsobariQ, MilQuant, Mascot, and Proteome Discoverer using an Orbitrap CID-HCD dataset. Peptides from were labeled with iTRAQ 4-plex reagents at relative amounts of 1:1:2:5 (114:115:116:117). Proportions of proteins ratio deviated by less than 30% from the expected ratios are presented. (B) On a Q-TOF dataset where equal amounts of sample were labeled, protein abundance ratios was deducted by MilQuant, and the distribution of relative errors of these ratios is presented. (C) MilQuant was performed on a large-scale iTRAQ 8-plex Orbitrap HCD dataset. The protein abundance ratios were compared with the reported ones produced by Proteome Discover and showed high level of correlation. (D) The reporter ion peak intensities from a large-scale Q-TOF dataset were computed by MilQuant and presented high level of correlation with ones in the original report.

percent of all quantified proteins presented relative errors greater than 30%, while more than 60% were quantified with relative errors of less than 10%. MilQuant showed similar quantitation accuracy to Mascot and IsobariQ, further demonstrating its capacity as an accurate software tool for data generated by both Orbitrap and TOF mass spectrometers.

To further demonstrate the validity of MilQuant, we used two large-scale datasets from previous reports. The iTRAQ 8-plex HCD dataset was collected by LTQ-Orbitrap, using HCD MS/MS spectra for both identification and quantitation, and the iTRAQ 4-plex CID dataset was collected by QSTAR, a Q-TOF mass spectrometer [22]. Different identification algorithms could result in different identification confidence assigned to the same spectra, which in turn lead to different sets of high-confidence PSM hits [23], and thus alter the list of reporter ion intensities that were later combined into abundance ratios, therefore leading to a different version of peptide/ protein quantitation results. To highlight the performance of the quantitation aspect, we extracted PSM hits provided in the original studies and imported them into MilQuant as identification input and investigate the consistency between MilQuant and reported results. We compared the protein ratios and reporter ion peak intensities from the two datasets, respectively. The values should be the same for every protein or reporter ion peak, but since not all details of normalization and ratio combination algorithm used to generate the original results were reported, the possibly different computation used in MilQuant (Supplementary methods) could result in deviation from the original reports. However, despite this possible source of inconsistency, the protein abundance ratios and reporter ion peak intensities showed high correlation between MilQuant and reported results (Fig. 3C, D, Tables S3, S4), further demonstrating MilQuant's validity and applicability in various types of raw MS data.

While dissociation methods such as ETD, PQD, and HCD are all available for reporter ion peak generation and collection in LTQ-Orbitrap mass spectrometers, the former two has certain limitations. Although PQD provides the possibility of quantitation using an LTQ mass analyzer, these two methods both produce fragment ion peaks of relatively

low intensity [7,8], which generally increases relative error and variability of abundance ratios. Also, the special fragmentation pattern in ETD leads to reduced number of multiplex channels [3]. Therefore, in current practice HCD is the most popular choice for isobaric tagging-based quantitation. The duty cycle is usually much longer than CID, but the high collisional energy can induce both intense reporter ion peaks and rich peptide sequence information. However, optimization of these two aspects seems to go opposite ways in terms of level of collisional energy. A very intense collision could produce more intense reporter ion peaks, which implies better data quality for quantiation and reduced duty cycle [24], but could also lead to little relevant information for identification, as is the case in the in-house gel slice dataset that used a normalized collisional energy of 70% (Fig. S7, Table S5). Identification can be achieved at a lower energy level, but is still outformed by CID largely due to the longer duty cycle and hence reduced data collection frequency [9,14]. To address this issue, a dual scan scheme has been introduced, which uses high-energy HCD solely for quantitation while relying on CID for identification. As the duty cycle of HCD is much longer, the parallel CID that takes place in LTQ does not prolong the whole duty cycle of the dual scan scheme, and is hence "free of cost" in terms of collection frequency. Moreover, since different dissocation methods specialize in identification of different types of precursors, this scheme can be further developed into a decision-tree based method selection mechanism, such as a CID/ETD-HCD scheme, which increased the number of peptide identifications by ~20% [24]. This could lead to complex data structure, but MilQuant is sufficiently generalized to process such datasets.

The current version of MilQuant is able to process data from iTRAQ and TMT tagging and using MS/MS spectra for quantitation. It could also be extended to other isobaric tagging techniques, as long as users provide the mass ranges for reporter ion peaks extraction as well as correction factors for isotopic impurities. Another functionality extension of MilQuant aims to support quantitation data where reporter ion peaks are recorded in $MS^3$ spectra, a novel MS approach that has been reported to almost completely eliminates ratio interference during data collection [25]. Finally, MilQuant makes it easy to use data interpretation tools for further data mining. For instance, with the uniform analysis workflows enabled by MilQuant, researchers can apply the desired analysis protocol on isobaric tagging datasets in various formats and process the results with Rover, a quantitation integration tool that increases the numbers of quantified proteins and peptides by combining datasets [26].

## 5. Conclusions

MilQuant offers a generic solution to the data analysis of isobaric tagging-based quantitation. It is designed as a highly customizable pipeline, with each step of which exporting various outputs that further promote the convenience of user customization. Added with its wide raw data compatibility, effective intrinsic algorithms, extendibility to novel techniques, and user-friendly interfaces, MilQuant serve as a flexible, free, yet full software solution to researches that employ the isobaric tagging techniques for quantitative proteomics researches.

## 6. Data access

The raw files of the HCD datasets used for performance comparison, database search files, and quantitation results can be downloaded from the ProteomeCommons.org Tranche system (http://www.proteomecommons.org). The Q-TOF CID datasets can be accessed at the PRIDE database (http://www.ebi.ac.uk/pride/), accessions numbers 9266–9283 (same-same dataset) and 16283–16284 (large-scale dataset).

Supplementary materials related to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.06.028.

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