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VIEWPOINT

Is label-free LC-MS/MS ready for biomarker discovery?

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Abstract

Label-free LC-MS methods are attractive for high-throughput quantitative proteomics, as the sample processing is straightforward and can be scaled to a large number of samples. Label-free methods therefore facilitate biomarker discovery in studies involving dozens of clinical samples. However, despite the increased popularity of label-free workflows, there is a hesitance in the research community to use it in clinical proteomics studies. Therefore, we here discuss pros and cons of label free LC-MS/MS for biomarker discovery, and delineate the main prerequisites for its successful employment. Furthermore, we cite studies where label-free LC-MS/MS was successfully used to identify novel biomarkers, and foresee an increased acceptance of label-free techniques by the proteomics community in the near future.

There is no doubt that any proteomics study needs to make the most out of an often limited and sometimes painstakingly acquired sample cohort, requiring accurate determination of both identity and quantity of the analytes from discovery to validation. This requires a highly reproducible and stable analysis workflow, capable of achieving accurate quantification as well as deep coverage of the investigated proteome. Currently, LC-MS/MS is the most flexible technique available for multiplexed protein quantification, and is the go-to setup to maximize proteome coverage. Deep coverage can for example be achieved by sample prefractionation or through depletion of high-abundance proteins. While the qualitative characterization (identification) of the proteome is by no means a trivial task, scoring matches of MS/MS fragments to database sequences is a task which has reached relative maturity, even if there exists a large number of different algorithms for performing the task [1]. In comparison, the peptide and subsequently protein quantification strategies available for an LS-MS/MS setup show far larger diversity, resulting in larger differences in both accuracy and dynamic range.

LC-MS/MS quantification strategies can be divided into workflows employing isotopic labels (predominantly metabolic [2] and isobaric [3, 4]) and label-free (spectral count and precursor-based) approaches. Metabolic labels are isotopic labels that are introduced through metabolic assimilation by cultured cells, and have the advantage of minimizing variation introduced by sample processing, if samples are mixed before processing. The main drawback of metabolic labeling is that it is limited to samples undergoing active protein synthesis, which makes it effectively impossible to use in its original version for clinical samples. To this

end, super-SILAC [5] has been introduced where a mixture of SILAC labeled cells are used as spike-in standard to be able to interrogate human tumor tissue. The value of such a study depends heavily on the design of the cell mixture, as even a combination of different cell lines may not accurately reflect the complexity of human tissue, and could reduce the effective proteome coverage. A label-free approach in such cases could be beneficial, as it does not impose any requirements about a reference sample. Furthermore, in recent work [6], label-free quantification was shown to outperform SILAC in terms of both proteome coverage and dynamic range.

Isotopic labels introduced during sample processing are also popular, and in particular isobaric labels have gained widespread used due to excellent multiplexing capabilities. However, several studies have shown a limited dynamic range in isobaric label workflows in comparison to the label-free approaches [7-9]. This tendency could be partially attributed to the co-isolation of multiple precursors for MS/MS fragmentation, which can be alleviated by using MS3 for quantification [10].

From a theoretical perspective, as MS spectra on an Orbitrap is currently restricted in the number of ions that are measured in each spectra, it is evident that dynamic range will be sacrificed in all types of multiplexing scenarios. Furthermore, the more densely populated the LC-MS run is, the higher the risk of ion suppression effects.

In summary, label-free quantification can be considered to have both higher dynamic range as well as proteome coverage capabilities in comparison to labeling techniques. Furthermore, label-free strategies are scalable to any number of samples in a straightforward manner. While workflows employing labels are scalable as well, they typically rely on a pool to enable within LC-MS/MS map comparison before scaling up to between-sample comparisons.

Despite these advantages, label-free workflows are in many cases still not the primary choice. We believe this is attributed to two main reasons: Firstly, the insufficient resolving power of previous generations of spectrometers and secondly, label-free quantification has been used as a term combining two very different quantification strategies, namely precursor-based quantification and spectral counting (Figure 1). While the above discussion concerning labeling versus label-free strategies is valid for both types of label-free quantification, spectral counting cannot resolve small abundance differences. Although recent developments in spectral counting has incorporated the intensities of the MS/MS peaks as well as other standardizing factors into the quantitative measurement e.g. [11, 12], there is a risk of introducing bias into the analysis due to the dynamic exclusion settings of the instrument which could favor certain elution patterns if not optimized. Furthermore, spectral counting suffers from a drawback common to all MS/MS-based quantification, namely the coupling of identification and quantification. This prevents the analysis of yet unidentified peptide patterns, which may be interrogated through repeated analysis of the sample using directed proteomics [13] (Figure 2). We therefore advocate label-free precursor-based quantification as the method of choice and is what we refer

to in the following text with the term label-free quantification unless stated otherwise.

Label-free workflows seem to not be used extensively due to the perceived risk of introducing spurious variation into the workflow due to the high demand of reproducible experimental procedures as well as relatively extensive data processing. It should however be noted that reproducible sample and data processing is key to any successful biomarker study, and while using labels will decrease some of the technical variation, it does not automatically reduce systematic differences, i.e. bias. Furthermore, if a study comprises more samples than the possible multiplexing capabilities of current labels, the same general considerations need to be taken. Also, irreproducible fractionation poses large challenges for any data processing strategy, as it is non-trivial to combine quantities from different fractions. For label-free quantification, the newly introduced MaxLFQ algorithm [14] proposes a solution for this by matching adjacent fractions between samples, and a new normalization approach. However, with MS in data-dependent mode, in combination with recent advances in sample preparation, it is now possible to identify 4000-6000 proteins in cell lines without pre-fractionation [15-17], considerably simplifying the experimental process and minimizing the introduction of technical variation.

As LC-MS/MS runs inevitably will be compared in label-free workflows, another key requirement is to keep system performance as constant as possible. One should thus have a system in place to monitor the performance of the LC-MS/MS setup. Typically this would involve repeated injections of a standard sample, and

preferably an automated readout. Several such quality control systems are becoming available and were recently reviewed [18, 19]. Preferably, monitoring should involve both MS and MS/MS level readouts, and retention time, number of identified MS/MS, number of detectable MS peptide peaks in order to track possible fluctuations. Nevertheless, some instrument fluctuations can be compensated for by using data normalization, as is discussed below.

Proper data processing is another key to successful protein quantification using LC-MS/MS [20]. There are multiple platforms for processing of label-free LC-MS/MS data, reviewed in recent publications [21, 22]. Precursor-based label-free quantification consists of two main steps: feature detection and alignment, where the former concerns extracting the peptide quantities from the LC-MS maps and the latter the process of correcting for elution time drifts between the maps and so facilitating the comparison of peptide and subsequently protein abundance. Whereas the feature detection step shares many elements with especially SILAC peak picking, alignment is a process unique to precursor-based label-free quantification. Although alignment adds to the complexity of the data processing, the step propagates identifications between peptides, contributing to the relatively high proteome coverage displayed by label-free quantification. For the example dataset described below, we found a sequence increase of on average 20% per LC-MS map. With the advent of spectrometers with considerably higher sequencing speed, capable of identifying almost all peaks in a spectrum, alignment will become obsolete or at the very least trivial. The label-free data processing will therefore be reduced to a feature detection stage not dissimilar to what is already performed for SILAC, but with the advantage of no

limitation in sample type and numbers. Naturally, given this type of high-speed spectrometer, the workflow presented in Figure 2 will also become superfluous and it could be argued that MS/MS-based quantification should in this case be utilized, as the proteome coverage differences will be more or less eliminated between the different quantification strategies. The problem of co-isolation as discussed previously however still remains to be fully resolved and we maintain that the utility of label-free quantification will only increase as resolution and sequencing speed increases.

Normalization of abundance data is a post-processing step, which to some extent can compensate for variations in instrument performance and differences in sample amounts, which are major issues for label-free quantification. As biomarker discovery studies in some cases inevitably will involve data acquisition over long time periods, it may be impossible to keep instrument performance stable. To illustrate this, we re-analyzed two samples consisting of spike-in peptides in cell-lysate background from a recent study [20]. New data acquisition was performed 22 months after the original analysis, and the LC-MS/MS system had undergone major maintenance in between these runs. To simulate further complications in the form of differing sample amounts, we halved the injection volumes for some of the new runs. The old and newly acquired data were then processed through a workflow named MSIP in the original publication, which includes msInspect for feature detection [23], peptide identification using Mascot (http://www.matrixscience.com), alignment [24] and further data processing in the Proteios Software Environment [25], with settings as in the original publication, except that X!Tandem searches were omitted for

simplicity. As illustrated in Figure 3a, the total intensity of the identified peptide features varied greatly between the different sample injections. The difference between the two samples (named dil8 and dil9 in the original study) is in the amount of spike-in peptides, with an approximately threefold difference, which one would expect to be readily detectable by student's t-test, considering the number of replicate injections for the two samples. However, due to the large coefficient of variation (CV), no significant differences could be detected between the samples with a 5% FDR threshold. We then normalized the data in a newly developed tool [26], to analyze if normalization could improve the data characteristics. As seen in Figure 3b, all normalization methods tested decreased the intra-sample CVs drastically. Furthermore, we looked at the batch effect between old and new acquisitions, and there was a clear batch effect seen before normalization (Figure 3c). However, normalization improved the situation considerably, and removed much of the batch effect, although some still remained (Figure 3d). The normalization effect was also evident on the results of the statistical test for significance, as LOESS [27] normalization allowed for detection of 66 spike-in peptides as significantly differentially expressed between the samples, albeit with another 3 background peptides detected as false positives. It is still thus clear that normalization to some extent can improve these types of datasets.

An important factor, which cannot be compensated for by normalization, is that low sample volumes or decreased MS efficiency may hinder detection of low-abundance peptides when signal goes below the noise level. In our test data, the number of missing values was highest in the samples with halved injection

volume (approximately 10% more missing values than in any other sample in the batch). Thus, despite advantages gained by normalization, keeping system performance constant with high sensitivity, and reproducible sample processing are still major considerations to fully succeed with label-free quantification. As mentioned above, this is also the case for workflows employing labels.

An interesting question is whether it is better to restrict studies to a lower number of samples, which could be analyzed over a short time, or to extend studies over an extended period of time if samples are available. Of course biological variation is best compensated for by analyzing many samples, but if system variation increases, it may become difficult to detect more subtle biological differences. In the tested dataset, upon normalization, we found 57 differentially expressed true positive (TP) and zero false positive (FP) peptides when only processing the four replicates in the old batch, as compared to 69 (66 TP and 3 FP) in the full batch (both old and new samples). In this case it was thus still better to extend the sample numbers, even if system performance and sample volumes varied over time. Although these numbers may not be readily transferable to other projects, they serve as an indication of the feasibility of longitudinal label-free experiments.

As there are several requirements, which can be difficult to evaluate individually, we recommend performing a pilot experiment, before starting any study. Ideally two samples from different phenotypic groups should be selected, and these should be mixed in different proportions as previously described [28]. Replicate injections will help to give an idea about expected CVs, and linearity checks (F-

test) to evaluate quantitative performance. The proteome coverage can also be evaluated, and would further help in deciding about further sample separation or LC gradient length.

In recent years, an increasing number of publications show that label-free quantification is viable for large-scale biomarker studies; a table of publications where the findings from label-free quantification has been validated by orthogonal methods can be found in [29]. Two examples of where sound experimental design in combination with label-free quantification have resulted in successful biomarker discovery have been presented by the groups of Banks [30] and Umar [31]. In [30], Smith et al. did a comprehensive study on improving the outcome of renal transplantation, starting with a smaller scale pilot study for biomarker candidate selection and validating results on a larger study where the number of samples utilized was determined by statistical power analysis. Further follow-up was performed long-term, clearly displaying the viability of label-free quantification in a large-scale study in combination with sound statistical analysis. Liu et al [6, 31, 32] describe a robust label-free quantification pipeline for investigating laser capture microdissected tissue, with a focus on breast cancer. The pipeline was first presented in [32], showing high reproducibility both on quantification and identification level. In [31] a large-scale label-free study is performed on aggressive triple-negative breast cancer, developing a protein signature which could possibly spare 60% of patients unnecessary chemotherapy treatment. Independent samples were used for validation. Common to the above mentioned studies was the utilization of the MaxQuant [14, 33] software, which on our benchmarking dataset was shown to

produce good results [20]. An example of a recently published study [34] using super-SILAC for finding prognostic markers for breast cancer and using both targeted mass spectrometry and immunohistochemistry as validation. While the study is well executed with a large sample cohort, it is clearly stated that out of the 8750 identified proteins, 7800 of them were quantified, a ratio of 90% of quantification to identification. The question naturally arises whether these results would look different had a label-free quantification strategy been applied?

Given the examples above, we believe that label-free should now be the first hand choice for discovery biomarker studies, as long as the basic requirements can be fulfilled.

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Figure Legends

Figure 1

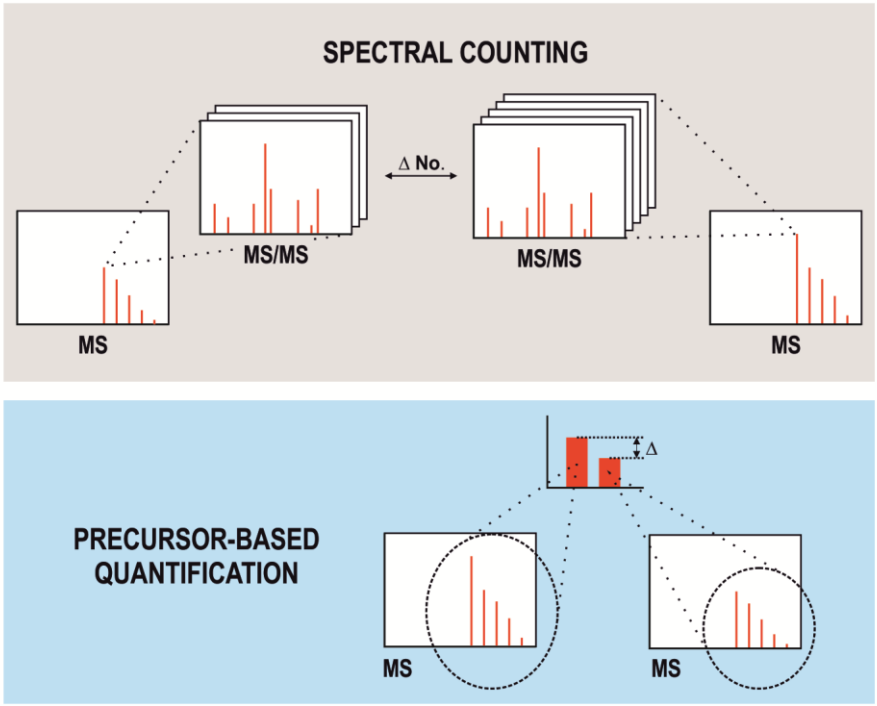
Overview of the two conceptually different label-free quantification techniques, based on either the number of times a peptide is subjected to a fragmentation even (spectral counting) or the summation of the peptide peaks along both mass-to-charge and retention time dimensions (precursor-based quantification), example only shown for one scan. Reprinted, with permission, from [35].

Figure 2

An overview of the label-free precursor-based workflow, showing both the possibility of further investigating interesting protein profiles as well as the different data processing steps and whether they are performed within single runs (intra-map) or between runs (inter-map).

Figure 3

Effect of normalization on samples analyzed at different time points and with different injection volumes. a) Total intensity of identified peptide features of the different runs. Samples 'Old' and 'New' were analyzed 22 months apart. Injection volumes as indicated. b) CV within samples dil8 and dil9 before (log2) and after normalization by different methods, performed in Normalyzer. c) and d) Fold change compared to mean value of each variable for old and new sample groups of dil8. c) log2 transformed data. d) LOESS-normalized data.



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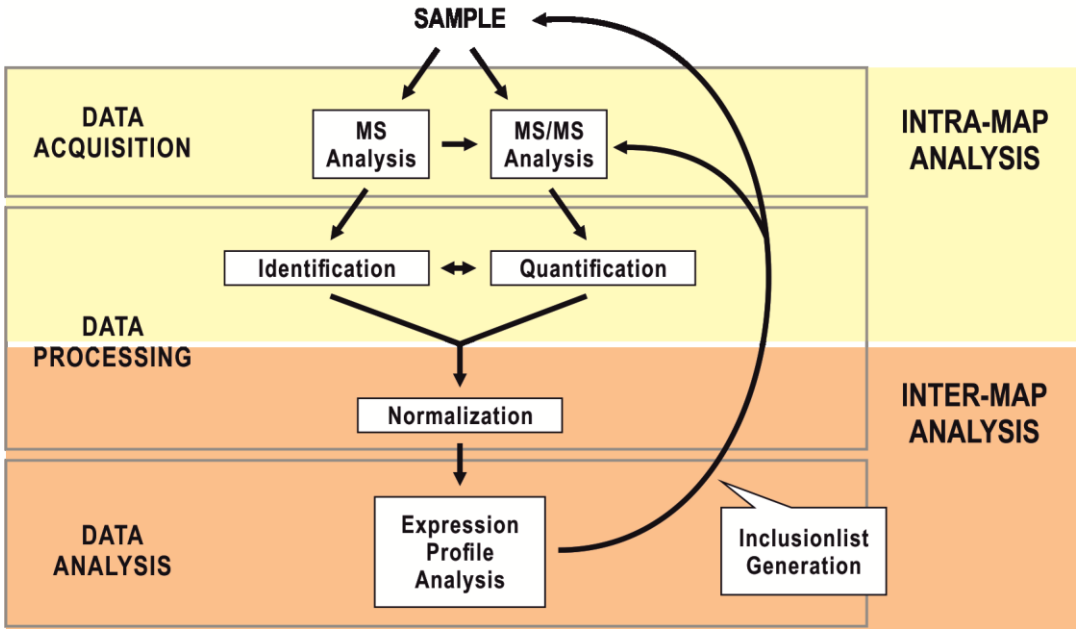

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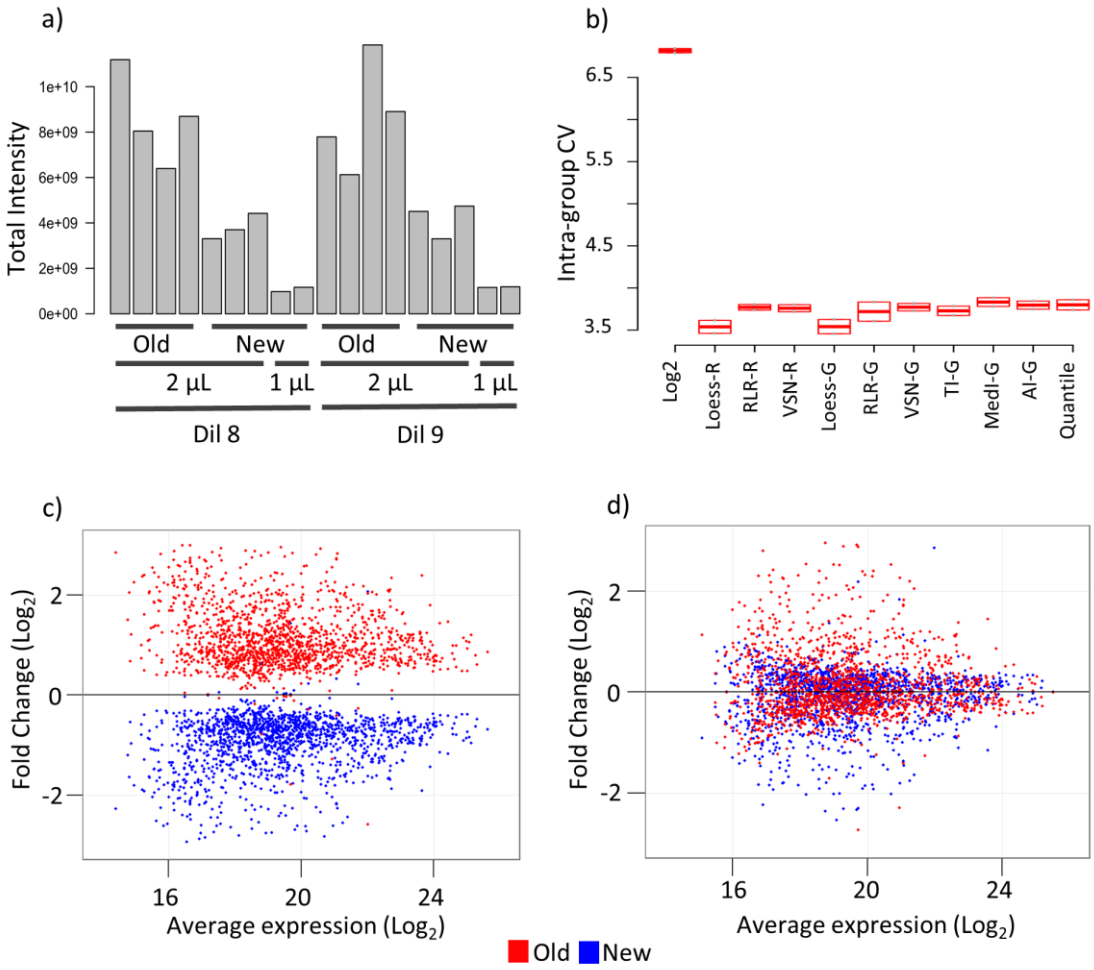

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