Using the entrapment sequence method as a standard to evaluate key steps of proteomics data analysis process

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Abstract

Background: The mass spectrometry based technical pipeline has provided a high-throughput, high-sensitivity and high-resolution platform for post-genomic biology. Varied models and algorithms are implemented by different tools to improve proteomics data analysis. The target-decoy searching strategy has become the most popular strategy to control false identification in peptide and protein identifications. While this strategy can estimate the false discovery rate (FDR) within a dataset, it cannot directly evaluate the false positive matches in target identifications.

Results: As a supplement to target-decoy strategy, the entrapment sequence method was introduced to assess the key steps of mass spectrometry data analysis process, database search engines and quality control methods. Using the entrapment sequences as the standard, we evaluated five database search engines for both the original scores and reprocessed scores, as well as four quality control methods in terms of quantity and quality aspects. Our results showed that the latest developed search engine MS-GF+ and percolator-embedded quality control method PepDistiller performed best in all tools respectively. Combined with efficient quality control methods, the search engines can improve the low sensitivity of their original scores. Moreover, based on the entrapment sequence method, we proved that filtering the identifications separately could increase the number of identified peptides while improving the confidence level.

Conclusion: In this study, we have proved that the entrapment sequence method could be a useful strategy to assess the key steps of the mass spectrometry data analysis process. Its applications can be extended to all steps of the common workflow, such as the protein assembling methods and data integration methods.

Keywords: Proteomics, Tandem mass spectrometry, Entrapment sequence method, Target-decoy search, Quality control

Background

The development of mass spectrometry has provided a high-throughput, high-sensitivity and high-resolution analysis platform for proteomics. Tandem mass spectrometry has become one of the most powerful technologies for protein identification, making possible the global protein profiling. Meanwhile, using the database searching strategy allows high-throughput identification of peptides and proteins in shotgun proteomics. Varied models and algorithms are implemented by different search engines, including the early produced engines SEQUEST [1], Mascot [2] and X!Tandem [3] as well as some newly developed engines, such as Comet [4], Tide [5], MS-GF+[6] and MS Amanda [7]. Then such quality control methods have been applied to achieve high reliability identifications as PeptideProphet [8–10], PepDistiller [11], Mfs [12], RockerBox [13], FDRAnalysis [14] and BuildSummary [15].
The target-decoy database search strategy is the most commonly used strategy to estimate false identifications in target database with the assumption that the number of false identifications in target database is equal to that in decoy database [16]. However, this strategy can estimate the false discovery rate (FDR) within a dataset rather than directly evaluate the false positive matches in target identifications.

In our previous work, we used the protein sequences from Archaea species as appended database for standard dataset analysis to avoid the ambiguous matches caused by the sequence similarity between control protein sequences and searched database sequences [17, 18]. Similar work had been published in Granholm et al.’s [19] and Vaudel et al.’s paper [20]. Granholm et al. suggested a semi-labeled method for evaluating the calibration of a given score function using dataset of known protein sample by searching the database composed of a small number of sample sequences and a large number of entrapment sequences. Vaudel et al. proposed constructing a database that contained both the sample sequences (true positive) and entrapment sequences (false positive) and proved that the Pyrococcus furiosus proteome can provide a method for detecting random hits (comparable to the decoy database).

All the above-mentioned work reminds us to introduce the entrapment sequence to target-decoy search strategy as a good supplement. By using different labels, we can separate the PSMs into different kinds and calculate the false matches in target identifications directly. Using the entrapment sequence as the objective standard (pure false positive), we assessed five database search engines and four quality control methods in terms of both quantity and quality. On the basis of the results of two sample identifications, we evaluated the false matches in target identifications directly.

In our previous work, we used the protein sequences (Homo20187, containing 20,187 sequences, downloaded on January 5, 2016). (3) Archaea protein sequences (Arc20825, containing 20,825 sequences, downloaded on September 21, 2016). We randomized the Archaea protein sequences ten times to get (4) The large entrapment sequences for LM3 dataset (Arc208250, containing 208,250 sequences). The composition of the three target databases is shown in Table 1. For Pfu dataset, the Pfu2045 was used as sample sequences and the Homo20187 was used as entrapment sequences. For LM3 dataset, the Homo20187 was used as sample sequences, while the Arc208250 and the Arc20825 were used as two different entrapment sequences. Then all target sequences were reversed to create the decoy database for target-decoy search strategy. The general view of the construction of searched databases is shown in Fig. 1.

Both Granholm et al.’s [19] and Vaudel et al.’s [20] work suggested sufficiently that large entrapment sequences should be used, and that the probability that a random match hits the sample database is negligible, but the best size hasn’t been examined. Here, about ten times as many entrapment sequences were used as sample sequences, which is a similar ratio to Vaudel et al.’s work. Also, we compared the tryptic peptides of all sample sequences and entrapment sequences. As shown in Table 1, the ratios of shared peptides are respectively low for three constructed databases (0.07%, 0.21% and 0.06%). Thus, very few positive PSMs should hit the entrapment sequences. A spectrum that matches both sample and entrapment sequences is considered a sample identification.

Database Searching
All mzML and MGF files were converted from raw files using the msconvert module [23] in the Trans-Proteomic Pipeline (TPP v4.7.0) [24]. The MS/MS peak list files were searched against the combined database using Mascot [2] (local server v2.3.2), Comet [4] (in Curx v2.1.16833 [25, 26]), Tide [5] (in Curx v2.1.16833), MS-GF+[6] (v10089) and X!Tandem [3] (TPP v4.7.0) [24]. The monoisotopic mass was used for both peptide and fragment ions with fixed modification (Carbamidomethyl, +57 Da) on Cys and variable modification (Oxidation, +16 Da) on Met. Tryptic cleavage at only Lys or Arg was selected. The miss cleavage number was set to be 1.

Quality control and protein assembling
Four commonly used quality control methods were used in this study, including BuildSummary [15], PeptideProphet [8–10], FDRAnalysis [14] and PepDistiller [11], all of which produced a rescoring of Mascot results for each PSM: BuildSummary’s ExpectValue, PeptideProphet’s...
probability, FDRAnalysis’s FDRScore and PepDistiller’s q-value. Comet and Tide results were processed by Percolator integrated in Crux, which gave a rescore of q-value. MS-GF+ and X!Tandem results were processed by percolator-converters (v3-00) followed by percolator (v2-08) for further quality control. The percolator tools can be downloaded from (https://github.com/percolator/percolator) [27]. In this study, we used MAYU for protein assembling [28]. Peptides less than 7 amino acids were not taken into account.

**False Discovery Rate and False Match Rate**

There are two formulas commonly used for false discovery rate estimation in target-decoy search strategy. One is for separated database search (formula (1)), and the other is for concatenated database search (formula (2)). $N_{\text{target}}$ and $N_{\text{decoy}}$ are the number of target and decoy matches, respectively.

$$FDR = \frac{N_{\text{decoy}}}{N_{\text{target}}}$$  \hspace{1cm} (1)

$$FDR = \frac{2 \times N_{\text{decoy}}}{N_{\text{target}} + N_{\text{decoy}}}$$  \hspace{1cm} (2)

As we introduced the entrapment sequences in the target database, the entrapment hits in filtered target identifications can be considered as false positive results. Thus, we defined a false match rate (FMR) to approximately estimate the false positive identifications under given FDR. The FMR can be calculated by formula (3), where $N_{\text{trap}}$ is the number of identifications matched the entrapment sequences in target hits.

$$FMR = \frac{N_{\text{trap}}}{N_{\text{target}}}$$  \hspace{1cm} (3)

**Results and discussion**

With the advance of proteome research, a growing number of database search engines as well as the subsequent quality control methods have emerged and played the key roles in the whole process of MS/MS data analysis. As shown in Fig. 1, using the entrapment sequences as a standard, we performed the evaluation of five database search engines’ original scores and reprocessed scores.
and four quality control methods in the two important aspects, quantity and quality.

**Evaluation of different database search engines based on both the original scores and reprocessed scores**

First, we used the Pfu dataset as a standard dataset to compare five search engines based on their original scores, Mascot’s ionscore, X!Tandem’s expect, Comet’s e-value, MS-GF+’s EValue and Tide’s XCorr. As shown in Additional file 1 Figure S1A-C, the MS-GF+ far outperforms the other search engines, and the use of the MS-GF+’s EValue allows significantly more identifications at all PSM, peptide and protein levels with the pre-defined FDR. The same trend has also been observed in the large LM3 dataset (Additional file 1: Figure S1D-F).

The original scores of search engines are usually of very low sensitivity. However, this flaw can be overcome when combined with the followed quality control methods by considering the distributions of target and decoy hits and reanalyzing the original scores. The SVM-based percolator algorithm has been proved to be an ideal QC method [11, 29]. Thus, we further analyzed Mascot’s results by PepDistiller [11] (a built-in Percolator classifier), X!Tandem’s results and MS-GF+’s results by Percolator [27, 30], Tide’s results and Comet’s results by Percolator integrated in Crux [25, 26]. All the above combinations can produce a q-value for each identification and be used for FDR calculation. As shown in Fig. 2, although the MS-GF+ combined percolator still performs best, other search engines can also perform quite well, especially in the large dataset (LM3 dataset) and at the stringent quality control level (protein level).

In previous studies, the performance of different search engines and quality control methods were assessed by the number of results identified with fixed FDR (e.g. 1% or 5%) estimated by target-decoy hits. The most productive tool or method is usually consider the best one, which is because only quantity is used as the criterion. Here, we introduced the entrapment sequence method as a complement to the target-decoy search strategy. Thus, we can use the entrapment hits to calculate the false march rate (FMR) to assess the quality of the results. As shown in Fig. 3 (also refer to Additional file 1: Figure S2), obviously, the FDRs determined by decoy hits remain stable (0.01 FDR of PSM, peptide and protein level respectively), while the FMRs vary with search engines and confident levels.

![Fig. 2 Plot figures of the numbers of PSM, peptide and protein identified by five search engines under the estimated FDRs on Pfu dataset (a-c) and LM3 dataset (d-f). The reprocessed scores of all five search engines are used](image-url)
In general, fewer entrapment hits occur in PSM and peptide identifications and in large dataset (LM3 dataset) than those in protein identifications and in small dataset (Pfu dataset). In most cases, the FMRs estimated by entrapment hits are roughly equal to those of FDRs estimated by decoy hits. But in some cases, the false matches represented by entrapment hits would far outnumber the expected ones, such as the Tide (FMR = 3.2%) and X!Tandem (FMR = 2.7%) searched results in Pfu dataset in 0.01 protein FDR condition (Fig. 3c), which would remind the researcher that more strict QC should be applied. Thus, we concluded that the entrapment sequence can be used as an internal scale for researchers to monitor their peptide or protein identifications at any time.

**Evaluation of four quality control methods**

As Mascot is one of the most widely used search engines, it has been improved to accommodate the MS/MS data generated by different instruments with different accuracy, and most quality control methods can handle its output result files. Here, Mascot’s searched files were used as inputs and reprocessed by four QC methods, including PepDistiller, BuildSummary, PeptideProphet and FDRAnalysis. As shown in Fig. 4, the percolator based QC method PepDistiller identified the most PSMs, peptides and proteins in both Pfu and LM3 datasets, and other three methods were not significantly different. The trends of FMRs of filtered results by different QC methods are close to the predefined FDR than those of search engines (Fig. 5). Using MAYU as the...
A protein assembling tool can help four QC methods to keep confident at the peptide and protein level, especially in the large LM3 dataset.

**Combining identifications of different search engines and quality control methods with an appropriate framework**

Varied models and algorithms that are implemented by different search engines and quality control methods, which make themselves mutually complementary and well-performing for different subsets of mass spectrometry data. Each search engine and QC method can uniquely identify some spectra (Additional file 1: Figure S3). Indeed, combining the results of multiple database search engines or QC methods can increase identifications, however, more false positive hits will be produced by uniquely identified results.

Take LM3 dataset as an example. Under 1% PSM FDR, the distributions of PSMs identified by one or several search engines or QC tools are shown in Fig. 6 and Additional file 1: Figure S4. Obviously, the FDRs and FMRs of peptides identified by one or two tools are much higher than those identified by three or more tools (Fig. 6a and Additional file 1: Figure S4A). If all these PSMs of five search engine are directly put together, there are 167,259 PSMs in total, resulting in 25.88% ~ 74.61% more hits than any single engine, but the FDR increases to 2.66% with the FMR of 2.61% too. Here we proposed an alternative integrated method in which further filterings were applied to the identifications according to their overlap conditions. We separated the PSMs into subgroups by the number of identified tools, and then filtered each subgroup hits to keep their sub-FDR lower than the predefined one (Fig. 6b and Additional file 1: Figure S4B). There are total 137,342 PSMs identified by this integrated method, resulting in 3.37% ~ 43.38% more hits than any single engine, but the FDR decrease to 0.40% with the FMR of 0.48%.

Thus, combining the results of multiple database search engines and QC methods with an appropriate framework would benefit the data analysis process, increase the numbers of identified peptides and improve the confidence level of identifications.

**Using a small size of entrapment sequences to evaluate the search engines and tools in large dataset**

As mentioned in Granholm et al.'s [19] and Vaudel et al.'s [20] papers, to efficiently separate correct PSMs from incorrect ones, the size of the entrapment sequences is supposed to be many times larger than the size of the sample sequences. However, the oversize database would greatly increase the search time while decreasing the total positive identifications. Thus, an appropriate size database is preferable in practical use.

![Fig. 4 Plot figures of the numbers of PSM, peptide and protein filtered by four quality control methods under the estimated FDRs on Pfu dataset (a-c) and LM3 dataset (d-f)](image-url)
Here, we used the original Archaea protein sequences (Arc20825) as a small size entrapment sequence and reprocessed the LM3 dataset. Then the similar results are gained as with large size entrapment sequence search (details are shown in Additional file 1: Figure S5 and S6). Thus, an easy way to use the entrapment sequence method is to randomize the sample sequences, label them and combine them with the sample sequence to construct a routine target-decoy database search, so that the entrapment hits included in each step can be used to provide a rough estimation of the confidence of the intermediate or final results.

Conclusions
In this study, we proposed a complementary use of target-decoy search strategy for evaluation of proteomics data analysis workflow. The labeled entrapment sequences are combined with the sample sequences to construct the target database for search, then the entrapment hits can be considered as false positive results and used to access the quality of proteomics data analysis tools. Based on this method, we assessed the two key steps of the mass spectrometry data analysis process, database search engines and quality control methods. Tested by both standard and experimental datasets, we found that the new search engine MS-GF+ and the support vector machine model based quality control method PepDistiller performed best in all evaluated tools, and the performance of search engines can be improved after the combination with efficient quality control methods. We also proposed an alternative integrated method for results from different tools. Filtering the identifications according to their overlap conditions, we can increase
the number of identifications and improve the confidence level at the same time.

Moreover, the entrapment sequence method could be an excellent strategy to assess all steps of the mass spectrometry data analysis process. Its applications can be extended to protein assembling methods, data integration methods and so on. By objective assessment of all steps of the common MS data analysis, we can standardize the analysis pipeline of mass spectrometry data.

Additional file

Additional file 1: This file contains supplementary figures, including Figure S1-S6. (PDF 477 kb)

Abbreviations

FDR: False discovery rate; FMR: False match rate; MS: Mass spectrometry; PSM: Peptide spectrum match; QC: Quality control

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Availability of data and material

The datasets analyzed during the study are available in iProX with the identifier IPX0000812000 (www.iprox.org).

Authors’ contributions

JM and KS conceived and designed the project. XF, LL and JZ collected the datasets, constructed the protein sequence database and implemented the evaluation workflow. CC assisted with the analysis of LM3 dataset. JM, KS and YZ made intellectual contributions to the whole project. JM and XF wrote the manuscript. All authors contributed to the editing of the manuscript, and all authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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