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Sequence analysis

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Analyzing marginal cases in differential shotgun proteomics

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ABSTRACT

Summary: We present an approach to statistically pinpoint differentially expressed proteins that have quantitation values near the quantitation threshold and are not identified in all replicates (marginal cases). Our method uses a Bayesian strategy to combine parametric statistics with an empirical distribution built from the reproducibility quality of the technical replicates.

Availability: The software is freely available for academic use at http://pcarvalho.com/patternlab.

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Supplementary information: Supplementary data are available at *Bioinformatics* online.

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1 INTRODUCTION

Shotgun proteomics describes a large-scale approach to analyzing complex peptide mixtures (i.e. mixtures originating from biological fluids, cell lysates, etc.). Briefly, the strategy is to perform protein digestion followed by peptide chromatographic separation online with tandem mass spectrometry (MS2) for protein identification (Washburn *et al.*, 2001). The study of complex mixtures is challenging in itself because peptides are under-sampled during data acquisition by mass spectrometry.

The combined nature of sample complexity, data acquisition methodologies and under-sampling is bound to generate considerable experimental variation. Indeed, one may expect to observe some 25% additional uniquely identified proteins when comparing two technical replicates of a complex mixture (Liu *et al.*, 2004). As we demonstrate below, this variation is largely due to peptide ions whose relative quantitation values lie near the detection threshold and therefore do not appear in all technical replicates (marginal cases).

One of the goals of proteomics is to distinguish between various states of a biological system according to protein expression differences. By directly applying common statistical approaches to pinpoint differentially expressed proteins without taking the necessary precautions that are inherently related to technical reproducibility, many marginal cases that are likely to be an artifact of chance may be included in the results and shadow important aspects. Moreover, many false negative cases may be lost.

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2 PROBLEM FORMULATION AND MODELING

Consider two biological states B_1 and B_2 and two experimental datasets, one containing replicates from state B_1, the other as many replicates from state B_2. We address the question of estimating the probability that a protein appearing in at least one replicate from state B_1 is differentially expressed with respect to state B_2, i.e. that it is found in none of the replicates from B_2.

If P is the protein in question, then our aim is to estimate the probability $P(H|D)$, where H stands for 'P is not detected in any replicate from B_2' and D for 'P appears in at least one of the replicates from B_1'. We assume throughout that the appearance of any given protein in a replicate from B_2 is subject to the same underlying laws governing its appearance in replicates from B_1, and moreover that it may occur in any of the replicates from B_2 independently with the same probability. This implies that the number of replicates from B_2 containing that protein is distributed binomially. Henceforth, we use the smoother, approximate formula of the Poisson distribution instead. Accordingly, the probability that the protein appears in u of the replicates from B_2 with mean λ is denoted by $\mathrm{Poi}(u, \lambda)$. In our estimates, we always choose the value of λ in reference to what is observed or hypothesized with respect to state B_1.

From a Bayesian perspective, we begin by estimating the prior probability $P(H)$ that protein P does not appear in any replicate from state B_2. If r is the number of replicates from state B_1 in which P is detected, then we set $P(H) = \mathrm{Poi}(0, r)$. Similarly, computing the desired probability, $P(H|D)$, requires that first we obtain $P(D|H)$ and $P(D|\text{not } H)$, that is, the probabilities that P is detected in at least one replicate from B_1 conditioned, respectively, on the fact that it does not or does appear in replicates from B_2. In order to estimate either probability, we first partition the B_1-replicate proteins into four groups of approximately the same size, each corresponding to one of the categories low, medium, high, or very high, according to the average signal of each protein (e.g. spectral count, peak area, etc.) over the replicates in which it appears. Let G denote the group to which protein P belongs. Our estimates of $P(D|H)$ and $P(D|\text{not } H)$ are relative to G, therefore specific to a certain range of average

signal. In what follows, we use f_t to denote the fraction of group-G proteins that occur in t replicates from state B_1.

We estimate $P(D|H)$ as the sum of probabilities of pairs of independent events. If n is the total number of replicates from either state, we consider one pair for each possible number t of replicates from state B_1, $t=1,\ 2,\ldots,n$. The two independent events for each pair are that a randomly chosen protein from group G appears in t replicates from state B_1, and that it appears in none of the replicates from state B_2. Thus,

$$P(D|H)=\sum_{t=1}^{n} f_t \mathrm{Poi}(0,t).$$

The case of $P(D|\text{not } H)$ is similar, but now the invalidity of H implies that we must sum up the probabilities that the randomly chosen protein from group G appears in u replicates from state B_2, for $u=1,\ 2,\ldots,n$. We then obtain

$$P(D|\text{ not } H)=\sum_{t=1}^{n} f_t \sum_{u=1}^{n} \mathrm{Poi}(u,t).$$

The desired probability, finally, follows from the Bayesian inversion formula,

$$P(D|H)=\frac{P(D|H)P(H)}{P(D|H)P(H)+P(D|\text{ not } H)[1-P(H)]},$$

and is henceforth used as a p-value for all proteins in G that appear in r replicates from state B_1.

3 DATA ACQUISITION

For evaluation of the above methodology, we used two shotgun proteomic datasets acquired by Fischer *et al.* (2010). Briefly, the authors employed Multi-dimensional Protein Identification Technology (MudPIT; Washburn *et al.*, 2001) to compare the A172 cell line in two biological states, here identified with the B_1 and B_2 states of Section 2. Each state was analyzed in triplicates (i.e. $n=3$). Relative quantitation was performed by spectral counting. A protein required a minimum of two peptides (thus, two unique spectral counts) to be considered.

4 RESULTS

Each of Supplementary Figures 1A, B and C shows a Venn diagram (VD) of identified proteins from B_1 and B_2 appearing in at least one, at least two, and all three replicates, respectively. Supplementary Figures 2A and B show VDs comparing uniquely identified proteins among the technical replicates from B_1 and B_2, respectively. Both Supplementary Figures 1 and 2 corroborate the great variability claimed by Liu *et al*. (2004).

The model described in Section 2 has been implemented as part of the PatternLab for proteomics suite (Carvalho *et al.*, 2008). Results on the biological states to which Section 3 refers are shown in Supplementary Tables I and II, respectively, to verify differential expression in state B_1 relative to state B_2 and conversely (i.e. reversing the roles of the two states in the discussion of Section 2). Clearly, proteins that are more reproducible (appear in more replicates) yield lower p-values.

The resulting algorithm was also incorporated into PatternLab's area-proportional VD module (Carvalho *et al.*, 2010). The user can now choose between generating VDs by filtering proteins that appear in at least a certain number of replicates, or by using the new approach through a user-specified p-value. The new option can be used to eliminate proteins that cannot be claimed to be statistically differentially expressed. Supplementary Figure 3 shows a VD that considers a p-value cutoff of 0.05 for the two biological states of Section 3, instead of the replicate-cutoff criterion used in Supplementary Figure 1.

5 FINAL CONSIDERATIONS

An alternative, simple strategy to pinpoint marginal proteins representative of a biological state is to consider only proteins that appear in a minimum number of replicates. Such an approach, however, is arbitrary and lacks proper foundation. The approach we have described, on the other hand, is well-founded and therefore amounts to a more refined method. It is useful especially in generating VDs, such as the one in Supplementary Figure 3, for the study of proteins that are representative of a given biological state. We note, in relation to VDs such as this, that uniquely identified proteins in the VD are not to be claimed as being unique to a state; instead, they are most likely differentially expressed.

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