IsoProt: A fully reproducible one-stop-shop for the analysis of iTRAQ/TMT data

Johannes Griss[1,2*], Goran Vinterhalter[3], Veit Schwämmle[4*]

1 EMBL-European Bioinformatics Institute, Wellcome Trust Genome Campus, CB1 10SD Hinxton, Cambridge, United Kingdom

2 Department of Dermatology, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria

3 Faculty of Mathematics, University of Belgrade, Studentski trg 16, 11001 Belgrade, Serbia

4 Department for Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark

* corresponding authors:

Johannes Griss, johannes.griss@meduniwien.ac.at, Telephone: 0043 1 40400 77150

Veit Schwämmle, veits@bmb.sdu.dk

Abbreviations

EuBIC	European Bioinformatics Community
FDR	False Discovery Rate
iTRAQ	Multiplexed Isobaric Tagging Technology for Relative Quantitation
PbA	*Plasmodium Berghei* ANKA
PSM	Peptide Spectrum Match
RMSE	Root-Mean-Square Error
TMT	Tandem Mass Tag

Abstract

Mass spectrometry coupled with isobaric labelling provides fast large-scale comparison of protein abundances over multiple conditions. To date, no one-stop-shop software solution exists that enables non-bioinformatics experts to carry out the full analysis of the acquired raw data with minimal intervention. Mostly, pipelines for such analyses are based on a combination of different software tools and in-house programs. In addition, different and often new versions of used tools and issues with the compatibility of apparently interoperable tools make it very difficult to ensure reproducible data analysis in the proteomics realm. We present a 100% reproducible software protocol to fully analyse data from one of the most popular types of proteomics experiments. The protocol uses only open source tools installed on a portable container environment and provides a user-friendly and interactive browser interface for configuration and execution of the different operations. An example use case is provided that can be used for testing and adaptation of own data sets. This setup will yield identical results on any computer analysing isobaric labelled MS data.

2

Introduction

Isobaric labelling has become one of the most common methods for quantitative mass spectrometry based proteomics experiments. A major advantage is that it allows researchers to multiplex samples and thereby reduce instrument runtime and eliminate variability caused by the mass spectrometer itself. The two methods currently available for these experiments Tandem Mass Tag (TMT, Proteome Science) and Multiplexed Isobaric Tagging Technology for Relative Quantitation (iTRAQ (1)) basically only differ in the reporter masses they generate but do not require dedicated software tools.

Even though isobaric labelling has become a standard method in many laboratories, no easy-to-use software solutions exist to analyse these data. This is particularly problematic when dealing with more complex experimental designs that include multiple runs on the mass spectrometer, such as multiple instances of differently labeled multiplexed samples. This causes many research groups to rely on unpublished in-house scripts to process their experiments which greatly hampers reproducibility. Even in the case of fully documented workflows, the use of different software versions or even only different versions of the underlying software libraries can dramatically influence the final results. Therefore, considerable effort is often required to replicate the computational processing of this common and widely used proteomics approach.

Workflow software suites and workflow managers, such as Proteome Discoverer (Thermo Scientific), OpenMS (2), and to a certain extent MaxQuant (3) with Perseus (4) allow the user to create a complete analysis workflow in a single software. An obvious but seldom executed step to increase reproducibility is to save this analysis workflow and deposit it in a public repository such as PRIDE Archive (5) together with the original data. However, full

reproducibility will only be achieved by creating a mirror of the used software pipeline together with its computational environment.

Furthermore, currently available workflow tools are rather complex to use or do not provide much flexibility in the statistical analysis of the data and supported experimental designs. For example, a standard OpenMS workflow to analyse isobarically labelled experiments can quickly grow to 15 different nodes all with their own settings. Moreover, to replicate the analysis, users have to install / buy the respective software and again ensure that all software versions (such as Proteome Discoverer node versions, or external OpenMS tools) are identical.

In an effort to simplify proteomics data analysis and provide fully reproducible data analysis workflows we launched the ProtProtocols project (https://protprotocols.github.io) under the umbrella of the European Bioinformatics Community (EuBIC). Based on the Biocontainers project (6) the protocols are shipped in containerized Docker images that include all necessary software tools. Docker containers are lightweight virtual machines that encapsulate all the software required for the protocol to run. This ensures that the version of all used software is linked to the protocol version and the user does not have to worry about installing any separate tools. Hence, 100% reproducibility can be achieved by using the same protocol version on any computer with a Docker environment.

Here, we present the IsoProt, a ProtProtocol designed for the analysis of isobarically labelled experiments. Next to a user-friendly web interface, it provides accurate statistical analyses over a wide range of common experimental designs.

Experimental Procedures

4

Software layout and implementation

General implementation

All software was installed in a Docker image to ensure full reproducibility on each computer system supported by Docker. To simplify the installation and usage of our protocols we created the free, open-source "ProtProtocol docker-launcher" (https://github.com/ProtProtocols/docker-launcher). It provides an easy-to-use graphical user interface that can automatically install the protocol (once Docker is installed) and launch the image. As it is written in Java it supports the major operating systems Windows, Mac OSX, and Linux. Therefore, many technical difficulties surrounding the use of Docker are hidden from the user.

The complete protocol is run through a Jupyter notebook (http://jupyter.org) corresponding to one web page in the browser. All relevant parameters can be set through common graphical user elements created through Jupyter widgets. Therefore, the user interface is highly similar to most available search engines. All steps are documented to facilitate usage. The complete source code as well as additional documentation of the protocol is freely available through https://github.com/ProtProtocols/IsoProt.

Proteomics software

IsoProt handles the entire analysis pipeline from mass spectra given as peak lists to a set of proteins that is differentially regulated between the given experimental conditions (Figure 1A). Herein, we used SearchGUI (7) and PeptideShaker (8) to perform peptide identification and validation, with MS-GF+ (9) as database search engine. Protein summarization and quantification is handled by R scripts based on the MSnBase R library (10). R scripts furthermore generate figures for quality control and perform statistical tests (LIMMA library, (11)) according to the experimental design.
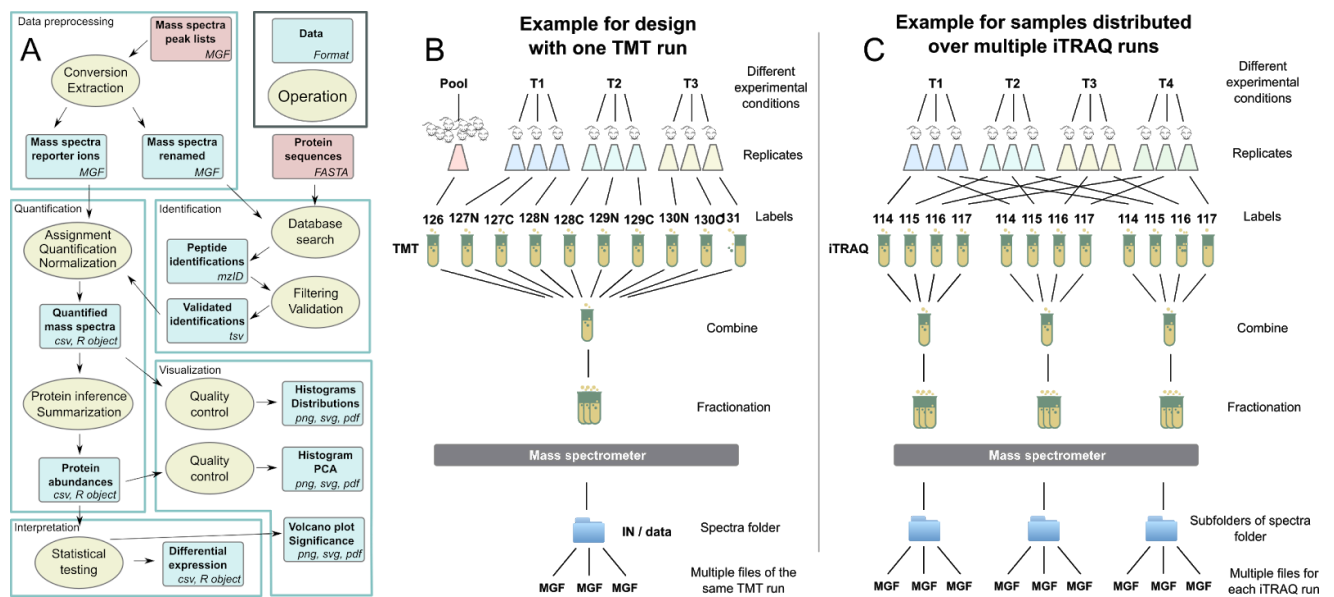
5

Figure 1: A Scheme of the entire workflow including operations (ellipsoids) and data given by type and format (squares). The annotation form and terms of the workflow follow to large extent the EDAM ontology (12). B+C Experimental designs and organization in folder structure for analysis in IsoProt.

Input files and parameters

Input files

The only files required for the analysis are mass spectra as peak lists (MGF format) and a FASTA file containing the protein sequences where we recommend the UniProt version of the FASTA format. Databases can already contain decoy sequences (following the SearchGUI instructions, http://compomics.github.io/projects/searchgui.html), otherwise the decoy database is created automatically. The files can be copied into the Docker file structure or directly mirrored onto the */data* folder which is automatically done by our docker-launcher application.

Analysis parameters

6

All parameters required for the data analysis can be changed through a graphical user interface integrated into the Jupyter notebook. In the first section, the user has to set database search related parameters such as precursor and fragment ion tolerance, the FASTA sequence database to use, the labelling agent used, and the fixed and variable modifications to consider.

Based on the selected labelling method and detected folder structure, the interface to enter the experimental design is generated. The protocol currently supports two setups: 1) all MGF files are placed in the input directory and are part of the same (fractionated) run or 2) if samples were spread over several runs on the mass spectrometer where MGF files from different runs are placed in different subdirectories per run (Figure 1B-C). The experimental design user interface now allows the user to enter names for the sample groups (for example "treatment" and "control"), names for the samples (one name per channel and subdirectory) and assign each sample to one of the groups. Most importantly, the protocol supports up to 20 sample groups and can thereby model complex experimental designs.

Finally, the user is asked to enter parameters related to the analysis of the quantitative data. Once all required information is entered, the search and analysis is directly controlled through buttons in the user interface.
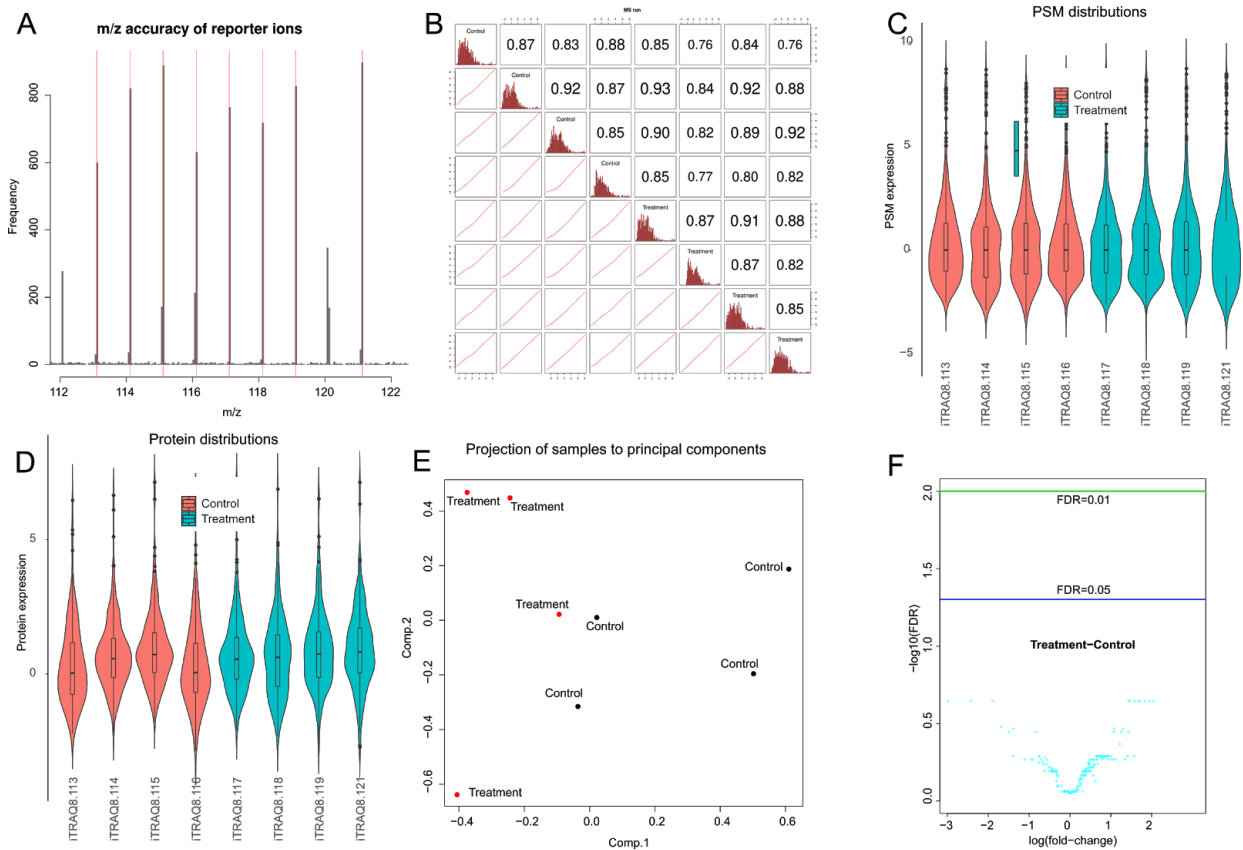
Figure 2: Examples of the visualisation and diagnostic plots created by IsoProt based on the shipped example data. A) The mass accuracy of all reporter ions is presented as a histogram. B) Correlation of reporter intensities for all channels. C) Distribution of estimated abundances on the spectrum level for all channels. D) Distribution of protein abundances for all samples. E) Principal component analysis of all samples based on the aggregated data highlighting the treatment groups. F) Volcano plot for a quick visualization of quantitative data and statistical results.

Output files and quality control

IsoProt provides figures and tables for the different steps of the analysis including peptide identifications, quantitative values of peptide-spectrum matches (PSMs) and proteins as well as a table for the statistical results from the significance analysis. Visual measures for quality control were implemented as R scripts and include total intensities of the reporter ion

channels for each sample, violin plots at different stages of the analysis, principal component analysis and volcano plots (Figure 2).

Test data sets

To evaluate the performance of our analysis workflow we processed the data from three publically available datasets. We downloaded the respective RAW files from PRIDE Archive (5) and converted them into the MGF file format using ProteoWizard's msconvert tool (13) when no MGF peak list files were available.

Benchmark dataset

D'Angelo *et al.* recently published a TMT benchmark dataset containing an experiment where 12 human proteins were spiked into an *E. Coli* background (14) using various concentrations (PRIDE Archive identifier PXD005486). D'Angelo *et al.* used this dataset to assess the number of proteins that were incorrectly identified as being regulated. As every protein was added using varying concentrations amongst the samples, a standard statistical analysis of the spiked-in proteins was not possible. Therefore, our analysis focuses on the accuracy of the derived quantitative estimates for the spiked proteins and the (unchanged) background *E. Coli* proteins.

The complete analysis was performed using IsoProt version 0.2. Spectra were identified using MSGF+ (9) through SearchGui version 3.3.3 (7). The precursor tolerance was set to 20 ppm and the fragment tolerance to 0.03 Daltons. 1 missed cleavages were allowed. Carbamidomethylation and TMT 10-plex of K,TMT 10-plex of peptide N-term were set as fixed modifications. Oxidation of M were set as variable modifications. PSMs were filtered at a target false discovery rate (FDR) of 0.01 using the target-decoy approach. UniProt E. Coli

sequences (version August 2018) and the spiked human protein sequences, also from UniProt, were used for spectra identification.

Quantitative analysis was done using the R Bioconductor package MSnbase version 2.7.1 (10). Protein summarization was performed using the medpolish method. Modified peptides were not used for quantitation. Only proteins with at least 2 identified peptides were accepted for further analysis. Differential expression was assessed using the R Bioconductor package limma version 3.34 (11).

Cerebral malaria pathogenesis

The study uses TMT6 labeling to compare mouse blood with different stages of cerebral malaria (d3, ECM) to non-infected mice (NI) (15). Four replicates of each of the three sample types were arranged in TMT6 sets and run separately. Peak list data files (MGF file format) were downloaded from PRIDE (PXD003772).

The analysis was again performed using IsoProt version 0.2 (see above) with the precursor tolerance set to 10 ppm and the fragment tolerance to 0.05 Daltons. 1 missed cleavage was allowed. Carbamidomethylation and TMT 6-plex of K,TMT 6-plex of peptide N-term were set as fixed modifications. Oxidation of M were set as variable modifications. PSMs were filtered at a target FDR of 0.01 using the target-decoy approach. SwissProt sequences from mouse (January 2018) were used for spectra identification.Only proteins with at least 2 identified peptides were accepted for further analysis.

Non-muscle invasive and muscle-invasive bladder cancer

The study compares tumor tissue samples from non-muscle invasive and muscle-invasive bladder cancer (16). MGF files were downloaded from PRIDE Archive (PXD002170).

The analysis was again performed using IsoProt version 0.2 (see above) with the precursor tolerance set to 10 ppm and the fragment tolerance to 0.05 Daltons. 1 missed cleavage were

allowed. Carbamidomethylation and iTRAQ 8-plex of K,iTRAQ 8-plex of Y ,iTRAQ 8-plex of peptide N-term were set as fixed modifications. Oxidation of M were set as variable modifications. PSMs were filtered at a target FDR of 0.01 using the target-decoy approach. Sequences from sp_human.fasta (SwissProt human proteome, January 2017) were used for spectra identification. Only proteins with at least 2 identified peptides were accepted for further analysis.

Results

IsoProt enables end users to run the full data analysis of iTRAQ/TMT experiments in a very straight-forward and reproducible way. The protocol can be applied to different experimental designs including multiple runs on the mass spectrometer and differently labeled multiple samples. This will be exemplified by a detailed description of the example workflow and evaluation of the results from three selected studies.

Apart from allowing data analysis through a few mouse clicks, the open layout of the protocol allows complex adjustments and modifications at all stages of the workflow.

A fully reproducible environment

The protocol can be run on any computer with a functional Docker environment, by just downloading and running the available Docker image. This is fully automated through our "ProtProtocol docker-launcher" tool (https://github.com/ProtProtocols/docker-launcher). Hence, the protocol avoids all possible platform- and operating system-specific installation issues and provides identical results independent of operating system, its configuration and computer hardware.

Every IsoProt release has a stable version number that points to a specific docker image. Therefore, by citing the used IsoProt version number it will always be possible to exactly restore the used analysis environment - including the versions of all used software tools.

Once the protocol has been executed, it is possible to save it, including all generated figures, as a standard HTML or pdf page. Therefore, the complete analysis workflow can be easily made available, for example at the time of review, and be viewed with a standard web browser. For an overview of the visualizations, see Figure 2.

Simple example workflow

Functionality and output of IsoProt can be tested using the available example data set which is small enough to run in under ten minutes on a standard computer. The database search via SearchGUI and validation via PeptideShaker result in a tab-delimited file containing detailed information of all PSMs. Search and output parameters are automatically saved for future reference. Additionally, a "methods" section is generated that can be included in a manuscript and describes all used settings. Each spectra file is processed separately to match and quantify PSMs that passed the identification FDR (default 0.01). The mass distribution of all matched fragment ions allows control for critical channels with inefficient labeling (Figure 2A). All PSM quantifications are saved in a separate file (AllQuantPSMs.csv).

The output of all files of each run on the mass spectrometer are merged, normalized and visualized for quality control. Violin plots of normalized PSM intensities compare the intensity distributions (Figure 2C). Channels with different distributions can identify problematic samples or changes within the entire proteome. Six different histograms counting PSM, peptide, protein and protein group numbers allows determining protein coverage and uniqueness by the available mass spectra. Similarity between samples is assessed through scatter plots comparing all quantified spectra from all ion channels (Figure 2B).

Using the default parameters, the PSMs are summarized to proteins using median summarization after outlier removal requiring a minimum of 1 PSM per protein. Other methods such as iPQF (17) are available. A violin plot of protein ratios versus mean of all channels shows whether the analysed samples exhibit similar distributions on the protein level (Figure 2D).

Quantifications from different runs (only one in the example) are merged and submitted to a principal component analysis (Figure 2E). With the scoring plot, one can look for similarity within replicates compared to different experimental conditions. Completely mixed samples are unlikely to provide differentially regulated proteins.

The example set quantified a total of 221 protein groups. LIMMA statistical tests did not find any regulated proteins with FDR < 0.05 which is in agreement with the original results. p-values and false discovery rates (p-values corrected for multiple testing) are visualized in histograms, volcano plots (Figure 2F) and a figure counting the number of differentially regulated proteins over a range of FDRs. The latter can be used to identify a suitable combination of the confidence threshold and the number of significant proteins. It is advised to keep FDR<0.1 as the number of false positives becomes critically high otherwise.

Performance tests by reprocessing public data

Benchmark dataset

D'Angelo *et al.* performed a comparison of different approaches to analyse TMT datasets (14). In their first dataset the authors spiked different concentrations of 12 human proteins into an *E. Coli* background. They used this dataset to assess the type-I error as the number of false positive proteins. Similar to the original study, we assigned the first five channels to one treatment group, and the second five channels to the second group. As expected, no

proteins were identified as being significantly regulated. The estimated log-fold changes of the *E. Coli* background proteins were all close to 0 (Figure 3A).

A

Fold change	2	4	20	40
Bias (our)	−0.6	−2	−11.4	−22.7
Bias (their)	−0.6	−1.8	−11.7	−21.9
RMSE (our)	1	2.1	11.8	23.5
RMSE (their)	1	1.9	11.2	22.3

Figure 3: A Log-fold changes of the *E. Coli* background proteins. This represents the expression of background proteins based on a comparison of the first five channels against the other five ones similar to the approach by D'Angelo *et al.* As expected, the estimated log-fold changes are all closely centered around 0. **B** Observed bias and RMSE of estimated fold-changes of the D'Angelo *et al.* benchmark dataset from our pipeline and the best-performing pipeline published by the authors.

Proteins were spiked twice using the same concentration in different channels and only once for the two highest concentrations. Therefore, only a single, or two replicate measurements at maximum are available when comparing two concentrations. This setup prevents a standard statistical evaluation, which is why we focused on the accuracy of the estimated fold changes using the same error measurements as in the original manuscript. Like D'Angelo *et al.* we measured the accuracy of our fold change estimates using the bias and

14

the root-mean-square error (RMSE). Across most spiked fold-changes, we observed a comparable bias and RMSE (Figure 3B).

For the highest spiked fold-change we observed slightly higher average error rates than D'Angelo *et al.* This is most likely caused by the fact the D'Angelo *et al.* imputed missing values by taking the lowest observed intensity of the given PSM across all samples. Thereby, missing values were automatically interpreted as very low expression. In our analysis, the measured abundance of the lowest concentrations was less stable. When we, for example, estimated the fold change of the two highest protein concentrations (also a fold change of 2), the bias is 0 with an RMSE of 0.2 improving the error rates dramatically.

While D'Angelo *et al.*'s imputation approach is valid if values can be expected to be missing not at random (ie. because of a concentration below the limit of detection) it is not valid for values missing (completely) at random (ie. because of inefficient labeling) (18). Therefore, for the spiked proteins D'Angelo *et al.*'s approach should only have been applied to cases were the lowest amount of proteins were spiked. In actual experiments, it is often unknown whether a value is missing at random or not which is why our pipeline is not using any imputation. The complete output of our pipeline can be found in Supplementary File 1.

Cerebral malaria pathogenesis
The authors investigated differences in the plasma proteome between healthy and malaria-infected mice (two stages). The available two TMT 6plex sets were considered to contain independent samples. IsoProt quantifies more protein groups (324 versus 289) when requiring a minimum of 2 unique PSMs and an identification FDR < 1%. For the further comparison, we restricted the IsoProt output to the uniquely identified 214 proteins (no peptides shared with other proteins).

15

In the original study, statistical testing was carried out separately for the two TMT runs,

yielding a total of 54 (more precisely 43 as 11 were detected in both runs) proteins found to

be differentially regulated between *plasmodium berghei* ANKA (PbA)-infected (d8

post-infection, labeled ECM) and non-infected (labelled NI) mice (Mann-Whitney *U* test,

p ≤ 0.001, no correction for multiple testing). We found a total of 41 differentially regulated

proteins (FDR < 0.01) and an overlap of only 20 proteins with the original study.

Given the rather different statistical testing, we looked into proteins that were not determined

as differentially regulated by either method. All but four proteins found differentially regulated

in the original study were quantified by IsoProt and showed similar abundances in both
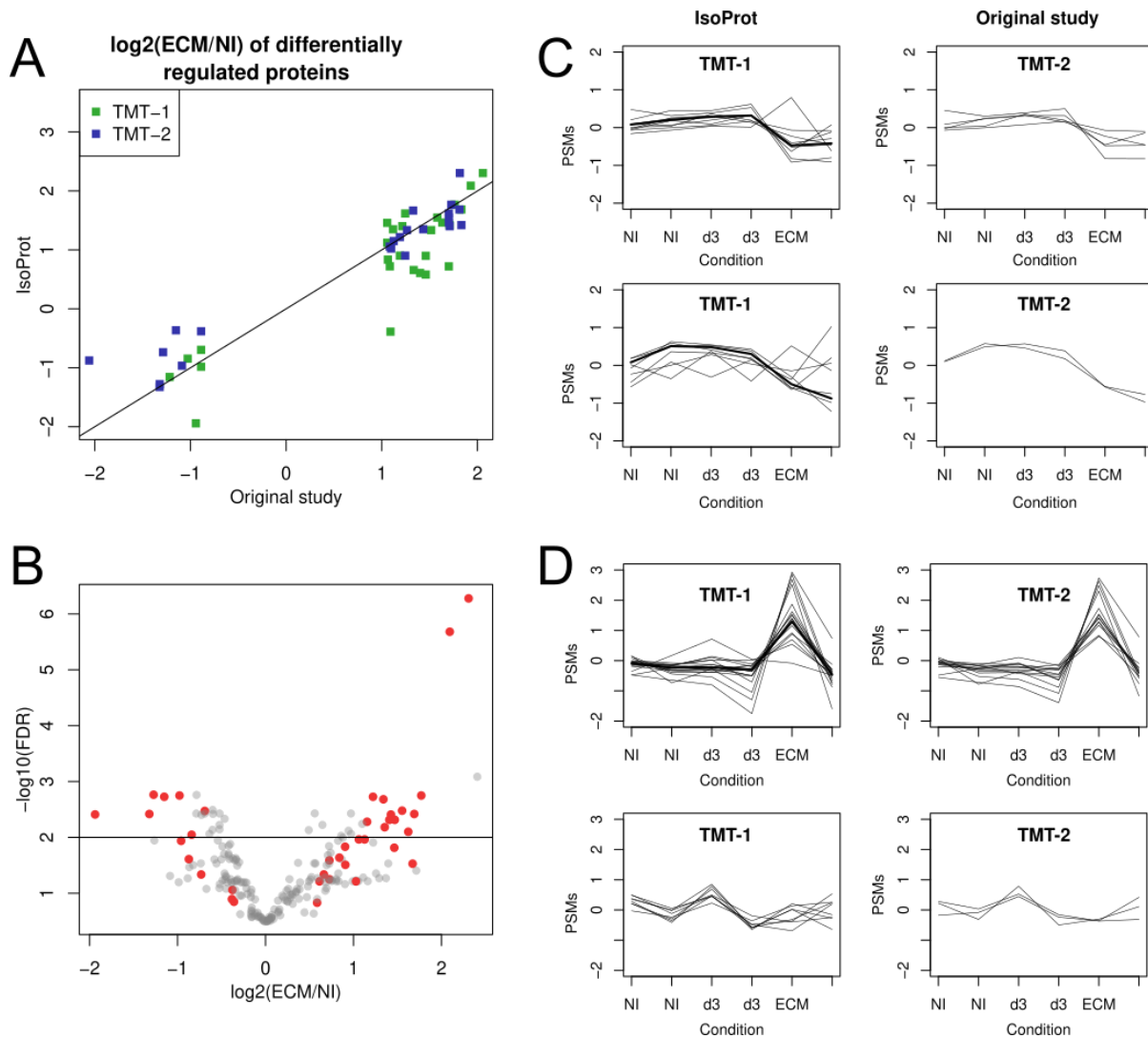
analyses (see Figure 4A)

16

Figure 4: A Comparison of fold-changes of proteins differentially regulated in the original study and IsoProt results. **B** Volcano plot of IsoProt results. Proteins found differentially regulated in the original study were labeled red. **C** Abundance profiles of Retinol-binding protein 4 (Q00724). **D** Abundance profiles of disulfide-isomerase (P09103).

Proteins only found significantly regulated in the original study were not found significant by IsoProt mostly due to low fold-changes in the quantitative analysis (Figure 4B).

We investigated the two proteins that mostly differ between the 2 types of analyses. Retinol-binding protein 4 (Q00724) was the protein with the lowest FDR within the proteins

17

found differentially regulated by IsoProt but not in the original study. Figure 4C shows PSM

measurements for the 2 TMT runs of this protein (scaled for better comparison).

Summarized protein abundances (thick lines) by median summarization with outlier removal

show that the PSMs of peptides with less differential behavior were removed. By merging

the observation of the two TMT runs, IsoProt gains more statistical power and thus provides

evidence for regulatory behavior of this protein.

On the other hand, protein Protein disulfide-isomerase (P09103) was the protein with the

highest FDR (least significant) of proteins found significantly changing in the original study

(TMT-1) but not by IsoProt (Figure 4D). Given only high abundances in one of the two ECM

replicates in TMT-1, at least manual interpretation would discard this protein from being

regulated (Figure 4D). The PSMs measured in the 2nd TMT-2 run confirm this observation.

The complete output of our pipeline can be found in Supplementary File 2.

Non-muscle invasive and muscle-invasive bladder cancer

IsoProt quantified 1,145 protein groups when restricting to a minimum of 2 unique peptides

and 1% FDR, compared to 1,092 in the original study (minimum of 2 peptides, Occam razor

principle for peptide inference and 1% FDR). Both analyses had an overlap of 662 proteins.

We then compared the mean log-ratios between the two cancer subtypes (four replicates

each). Despite only having different bioinformatics workflows, relatively large differences

were observed between the estimated log-fold changes (Figure 5A, Pearson's correlation of

0.78).

Statistical testing did show one differentially regulated protein (15-hydroxyprostaglandin

dehydrogenase, FDR < 0.01) after correction for multiple testing which has not been carried

out in the original study. When comparing uncorrected p-values, the majority of significant

proteins were different between the two studies (Figure 5B and C, colored points indicate

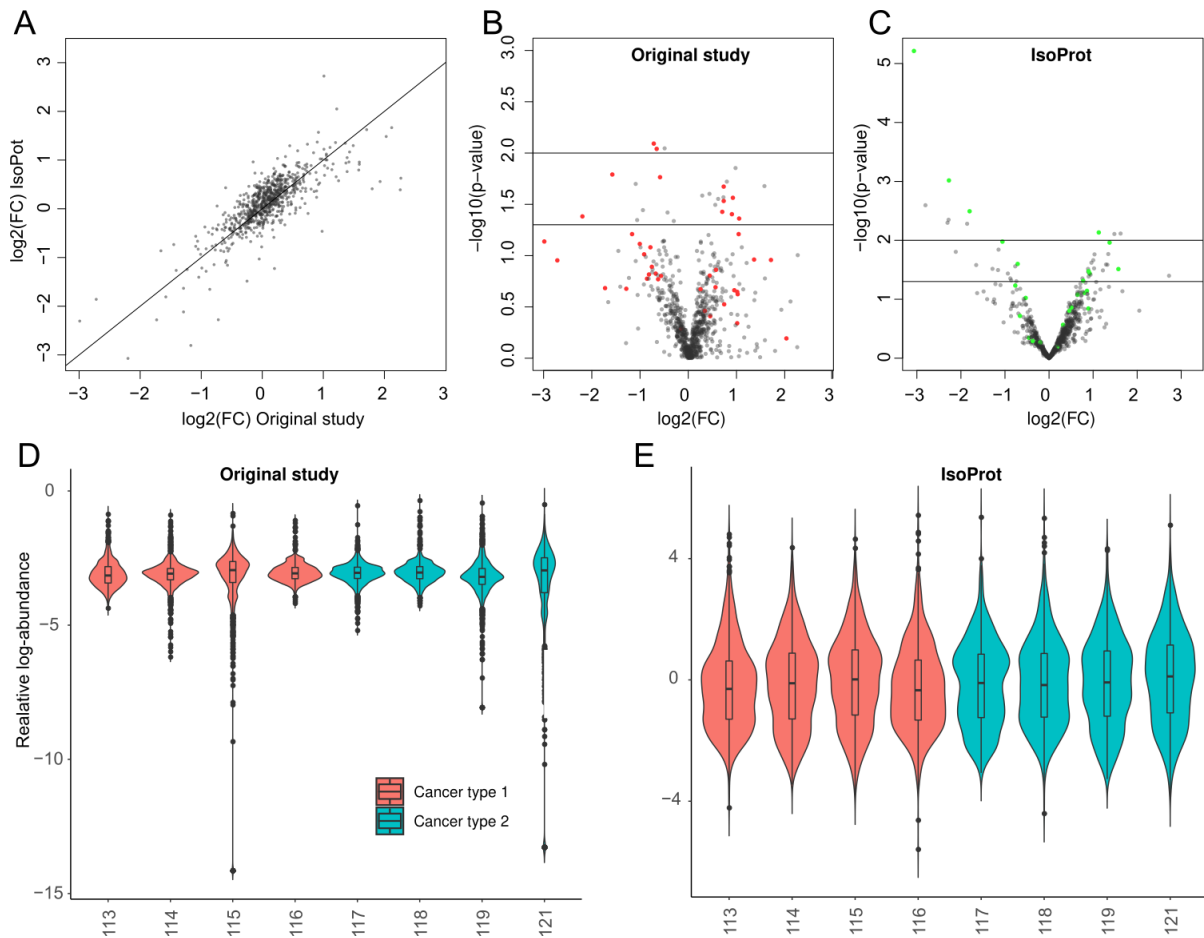p<0.05 in the other respective study).

Figure 5: **A** Comparison of log-ratios between IsoProt output and original study. Pearson's

correlation between both quantification: 0.79. **B-C** Volcano plots for results from statistical

testing in the original study (**B**) and in IsoProt (**C**). Colored points correspond to proteins with

a p-value below 5% in the other study, respectively. **D-E** Distribution of relative protein

abundances in original study (**D**) and IsoProt (**E**).

This striking difference in the statistical results can be explained when looking at the

distribution of protein abundances (Figure 5D and E). A deeper look into the original analysis

showed that the authors normalized the ratios between cancer subtypes after protein

19

summarization and averaging of replicates. The more common and in our opinion correct approach is to normalize the different channels (ie. individual samples) on the (measured) PSM or (aggregated) peptide level prior to the aggregated analysis of these measurements on the protein level and, most importantly, prior to merging any independent (ie. replicate) measurements. Strong deviations of individual channels which are visible on the peptide level were thus discarded in the original study. The complete output of our pipeline can be found in Supplementary File 3.

Discussion

While the availability of proteomics techniques is continuously increasing, researchers often do not have the required bioinformatic support to analyse the data. Here, we present a simple, statistically accurate protocol for one of the most commonly used quantitative approaches in proteomics. This should enable users to analyse even complex experimental setups with just a few mouse clicks.

Lack of reproducibility in general, and in bioinformatics workflows specifically is a growing concern. Seemingly small changes to a workflow, such as normalisation method details can have dramatic effects on the final result. Due to the many steps and settings that make a complex workflow, it's usually impossible to fully describe such a workflow in the methods section of a research paper. The IsoProt protocol allow users to save the complete analysis workflow as a simple HTML page which can then be submitted in addition to the classical methods section in a paper.

Finding exactly the same software version used in a paper is often a major obstacle when replicating bioinformatic analyses. Often, this older version is no longer compatible with the available operating system or is just altogether unavailable. By encapsulating protocols into

docker containers the complete setup including all software versions can be referenced through a single protocol version number. This allows anyone to replicate the results at any later stage without having to worry that older software might no longer work. Once a given version of the protocol is downloaded, users can be sure that it will behave in exactly the same way on all supported platforms.

The use of docker makes the protocol highly portable. Docker currently supports Windows, Linux and Mac OS making our protocol trully multiplatform. The fact that the protocol can be installed through a single command makes it trivial to move the setup from one machine to another. With our "ProtProtocol docker-launcher" tool the protocol can even be installed with the click of a single button. This should greatly reduce the effort in setting up a complex proteomics analysis environment and relieve the often already strained IT support.

Applying IsoProt to available data showed that subtle differences in the data analysis can lead to considerable differences in the final results. These differences are very difficult to spot during the review of a paper since (bioinformatics) methods can only be summarized in the manuscript. Therefore, if researchers are able to make the complete workflow available in an easy to read format such points can already be discussed at the time of review, and therefore increase quality and credibility of both the study and the journal.

All of these developments are available as free and open-source software. Thereby, we encourage other researchers to use the ProtProtocol infrastructure as starting point to develop their own analysis workflows and make them available to the community. All our tools are modularized and prepared to support and simplify such external developments. Since Docker has become an industry standard for containerized applications long-term support seems to be guaranteed for these developments.

21

In summary, we presented here an environment for fully reproducible data analysis and exemplified its power by hand of a fully functional software for the analysis of data from mass spectrometry experiments with isobaric labeling.

Acknowledgments

Data Availability

The complete software presented in this manuscript is freely available under a permissive open-source license at https://protprotocols.github.io.

References

1. Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., and Pappin, D. J. (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3, 1154–1169

2. Röst, H. L., Sachsenberg, T., Aiche, S., Bielow, C., Weisser, H., Aicheler, F., Andreotti, S., Ehrlich, H.-C., Gutenbrunner, P., Kenar, E., Liang, X., Nahnsen, S., Nilse, L., Pfeuffer, J., Rosenberger, G., Rurik, M., Schmitt, U., Veit, J., Walzer, M., Wojnar, D., Wolski, W. E., Schilling, O., Choudhary, J. S., Malmström, L., Aebersold, R., Reinert, K., and Kohlbacher, O. (2016) OpenMS: a flexible open-source software platform for mass spectrometry data analysis. *Nat. Methods* 13, 741–748

3. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification.

Nat. Biotechnol. 26, 1367–1372

4. Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat. Methods* 13, 731–740

5. Vizcaíno, J. A., Csordas, A., del-Toro, N., Dianes, J. A., Griss, J., Lavidas, I., Mayer, G., Perez-Riverol, Y., Reisinger, F., Ternent, T., Xu, Q.-W., Wang, R., and Hermjakob, H. (2016) 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* 44, D447–56

6. da Veiga Leprevost, F., Grüning, B. A., Alves Aflitos, S., Röst, H. L., Uszkoreit, J., Barsnes, H., Vaudel, M., Moreno, P., Gatto, L., Weber, J., Bai, M., Jimenez, R. C., Sachsenberg, T., Pfeuffer, J., Vera Alvarez, R., Griss, J., Nesvizhskii, A. I., and Perez-Riverol, Y. (2017) BioContainers: an open-source and community-driven framework for software standardization. *Bioinformatics* 33, 2580–2582

7. Barsnes, H., and Vaudel, M. (2018) SearchGUI: A Highly Adaptable Common Interface for Proteomics Search and de Novo Engines. *J. Proteome Res.* 17, 2552–2555

8. Vaudel, M., Burkhart, J. M., Zahedi, R. P., Oveland, E., Berven, F. S., Sickmann, A., Martens, L., and Barsnes, H. (2015) PeptideShaker enables reanalysis of MS-derived proteomics data sets. *Nat. Biotechnol.* 33, 22–24

9. Kim, S., Mischerikow, N., Bandeira, N., Navarro, J. D., Wich, L., Mohammed, S., Heck, A. J. R., and Pevzner, P. A. (2010) The generating function of CID, ETD, and CID/ETD pairs of tandem mass spectra: applications to database search. *Mol. Cell. Proteomics* 9, 2840–2852

10. Gatto, L., and Lilley, K. S. (2012) MSnbase-an R/Bioconductor package for isobaric tagged mass spectrometry data visualization, processing and quantitation. *Bioinformatics* 28, 288–289

11. Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., and Smyth, G. K. (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 43, e47

12. Ison, J., Kalas, M., Jonassen, I., Bolser, D., Uludag, M., McWilliam, H., Malone, J., Lopez, R., Pettifer, S., and Rice, P. (2013) EDAM: an ontology of bioinformatics operations, types of data and identifiers, topics and formats. *Bioinformatics* 29, 1325–1332

13. Adusumilli, R., and Mallick, P. (2017) Data Conversion with ProteoWizard msConvert. *Methods Mol. Biol.* 1550, 339–368

14. D'Angelo, G., Chaerkady, R., Yu, W., Hizal, D. B., Hess, S., Zhao, W., Lekstrom, K., Guo, X., White, W. I., Roskos, L., Bowen, M. A., and Yang, H. (2017) Statistical Models for the Analysis of Isobaric Tags Multiplexed Quantitative Proteomics. *J. Proteome Res.* 16, 3124–3136

15. Tiberti, N., Latham, S. L., Bush, S., Cohen, A., Opoka, R. O., John, C. C., Juillard, A., Grau, G. E., and Combes, V. (2016) Exploring experimental cerebral malaria pathogenesis through the characterisation of host-derived plasma microparticle protein

content. *Sci. Rep.* 6, 37871

16. Latosinska, A., Vougas, K., Makridakis, M., Klein, J., Mullen, W., Abbas, M., Stravodimos, K., Katafigiotis, I., Merseburger, A. S., Zoidakis, J., Mischak, H., Vlahou, A., and Jankowski, V. (2015) Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis. *PLoS One* 10, e0137048

17. Fischer, M., and Renard, B. Y. (2016) iPQF: a new peptide-to-protein summarization method using peptide spectra characteristics to improve protein quantification. *Bioinformatics* 32, 1040–1047

18. Lazar, C., Gatto, L., Ferro, M., Bruley, C., and Burger, T. (2016) Accounting for the Multiple Natures of Missing Values in Label-Free Quantitative Proteomics Data Sets to Compare Imputation Strategies. *J. Proteome Res.* 15, 1116–1125