**User Guide to:  
Site-To-Protein Normalization**

**in Multiplex Proteomics**

## Overview

This document serves as a guide to the demo on our GitHub (https://github.com/maxperutzlabs-ms/SiteToProteinNormalization\_in\_MultiplexProteomics) and for the workflow of site-to-protein normalization in multiplex proteomics in general, initially described in our publication (<https://doi.org/10.1016/j.mcpro.2023.100694>). The demo comes with its own dataset based on which the entire workflow can be experienced from start to finish. We recommend running the demo first before applying the workflow to your own data – this will the user familiar with the required data input, the script’s adjustable parameters as well as the intermediate and final data output. In this user guide, we will look at the required setup for running the workflow and then how to run it on the basis of the available demo dataset. Finally, there is a description of the generated output. Please note that as of now this workflow supports MaxQuant output only.

## Required Setup

# This workflow of site-to-protein normalization builds on the workflow of interference modeling in multiplex proteomics to provide the necessary input data. This demo therefore starts where the demo of interference modeling left off (please see GitHub repository: https://github.com/maxperutzlabs-ms/InterferenceModeling\_in\_MultiplexProteomics). That said, the required output of the first demo, named “modified\_PSM.txt”, is also available for download in the Demo folder of this GitHub repository, if you choose to skip the demo for the interference modeling part. For practical reasons, we assume that we just finished running the demo for interference modeling, which left us with the following data:

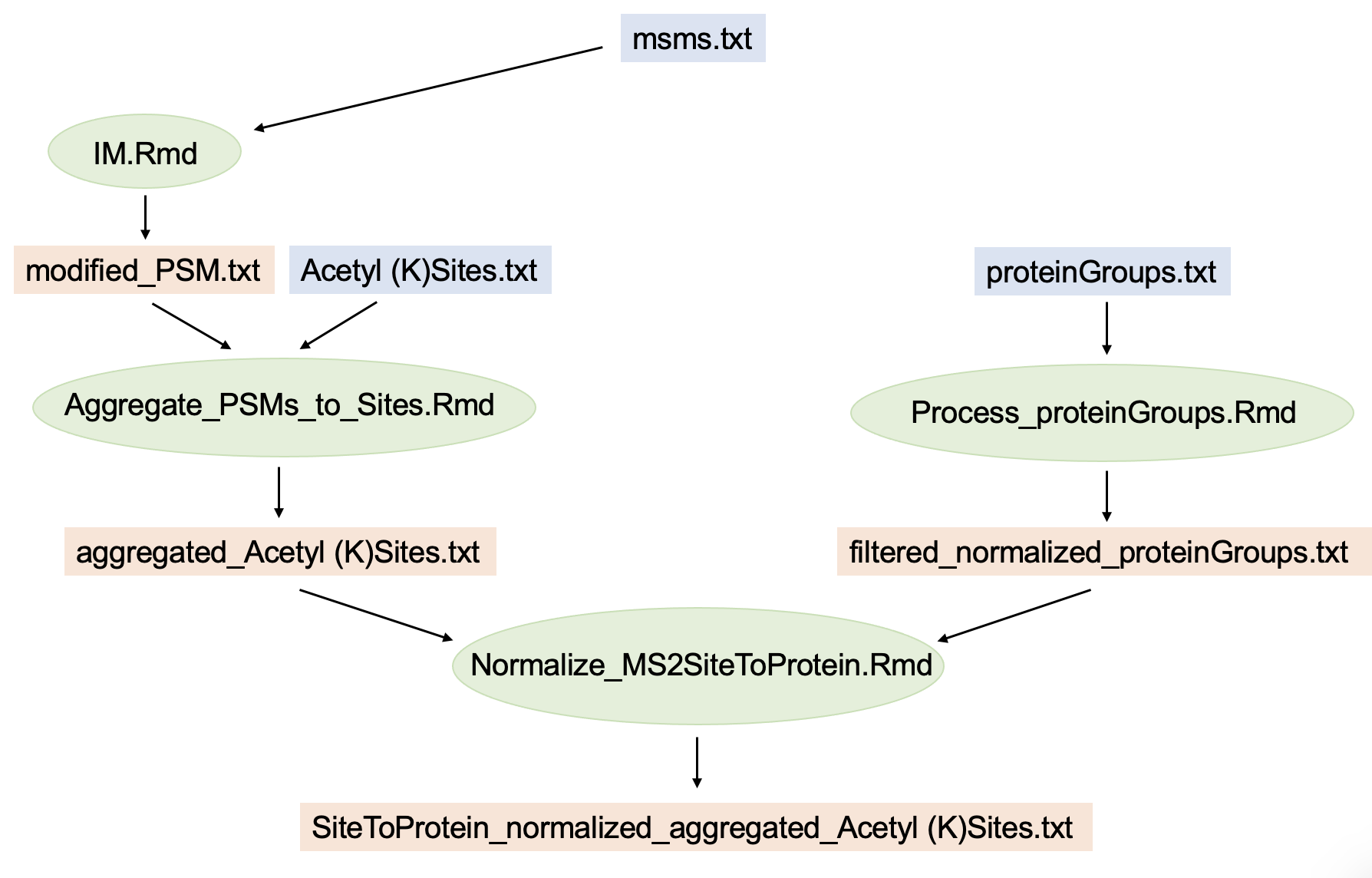
Graphical user interface, text, application, email

Description automatically generated ***Figure 1*** *- Overview of files and directories after running the interference modeling demo. This setup serves as the starting point for this demo.*

In the “Results” subdirectory, we find the main output of the interference modeling workflow named “modified\_PSM.txt”. If you did not run the demo for interference modeling, please download it now (it is stored in the Demo directory of this GitHub repository), and save it in a new folder named “Results”.

Before we continue, we should first consider where we are and what we actually want to do. The table “modified\_PSM.txt” contains PSM-wise information of quantified acetyl(K) peptides. Thanks to the interference modeling workflow, this table comes with some extra columns, including a column named EIL (Estimated Interference Level). This metric is crucial in unbiased site-to-protein normalization in multiplex proteomics, as it provides an estimate of the degree of interference/ratio compression of each PSM. As described in our paper (<https://doi.org/10.1016/j.mcpro.2023.100694>), if we can account for the varying degrees of ratio compression on both PTM site and underlying protein level, we have the means for unbiased site-to-protein normalization in multiplex proteomics.

The overall strategy of this workflow is to first carry over the relevant PSM-wise information (i.e., EIL values and reporter ion intensities) to the acetyl site level contained in MaxQuant’s “Acetyl (K)Sites.txt” via aggregation. Then we perform unbiased site-to-protein normalization by accounting for reporter ion interference on both site and protein level. In this demo, we will go through the workflow of normalizing MS2-quantified site abundances to MS3-quantified protein abundances (more specifically, FAIMS-MS3 quantified proteins with real-time-search on). This configuration requires us to adhere to the following steps as visualized in the README of the GitHub repository:



***Figure 2*** *- Overview of site-to-protein normalization workflow when normalizing MS2-quantified sites to MS3-quantified proteins. In blue: MaxQuant output; in green: R-scripts; in orange: Intermediate or final data output.*

# Arrows in the above diagram indicate input and output data directionality. Data input (taken from the MaxQuant search results) is shown in blue, data output is shown in orange, and R-scripts are shown in green. As we can see, the demo of interference modeling already concluded the first step, seen top-left in the above diagram, thereby producing “modified\_PSM.txt”. This is where we start for this demo. Looking at the rest of the graph, we see that we still have some steps ahead of us which requires some additional input data and R-scripts to run the entire workflow. Let’s first go over all the additional files shown in Figure 2 that we require:

* **Acetyl (K)Sites.txt** is a MaxQuant site table. It needs to come from the same database search result that generated “msms.txt”, because the ID columns of the two tables need to reference each other, thus allowing the aforementioned aggregation step. You can find this file on GitHub in the Demo folder.
* **proteinGroups.txt** is a MaxQuant protein table. It needs to come from the same database search that generated “msms.txt”. This table contains the intensity columns corresponding to MS3-based quantification of unmodified peptides (i.e. “proteome”). To ensure that MaxQuant produces these extra columns in the protein table output, the respective raw files were set as their own experiment during raw file configuration of the MaxQuant database search. You can find this file on GitHub in the Demo folder.
* **Aggregate\_PSMs\_to\_Sites.Rmd** is an R Markdown script to perform the aggregation of PSM level information contained in “modified\_PSM.txt” to site level information contained in “Acetyl (K)Sites.txt”. In short, the PSM-wise information of EIL values and between-sample-normalized reporter ion intensities is aggregated and carried over to the site level. This file is located in the main folder of the repository on GitHub. Its output is a file called “aggregated\_Acetyl (K)Sites.txt”, which will automatically be saved in the “Results” subdirectory.
* **Process\_proteinGroups.Rmd** is an R Markdown script to perform filtering and between-sample normalization etc. of protein reporter ion intensities contained in MaxQuant’s “proteinGroups.txt”. This file is located in the main folder of the repository on GitHub. Its output is a file named “filtered\_normalized\_proteinGroups.txt”, which will be saved in the “Results” subdirectory. In this demo, “Process\_proteinGroups.Rmd” further requires the otherwise optional input of an isotopic impurity matrix to correct for isotopic impurities of TMT labels. Fortunately, we can use the same impurity matrix already used in the interference modeling workflow named “**impurity\_matrix\_tmtpro.csv**”. If not yet contained in your working directory, you can download this file on GitHub in the Demo folder.
* **Normalize\_MS2SiteToProtein.Rmd** is an R Markdown script to perform the final step of site-to-protein normalization. This file is located in the main directory of the GitHub repository. It produces an output called “SiteToProtein\_normalized\_aggregated\_Acetyl (K)sites.txt”, which will be stored in the “Results” subdirectory.
* **functions\_Site\_To\_Protein\_norm.R** contains functions automatically sourced by the script “Normalize\_MS2SiteToProtein.Rmd”. This file is located in the main directory of the repository on GitHub.

Let’s download the required files mentioned above and put them into the directory where we run the demo. It should end up looking like this:

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***Figure 3*** *- Overview of required setup of all directories and files to complete this demo. Some of the files shown were produced or required by the Interference Modeling workflow and therefore not necessary to continue this demo.*

## Running the Program

We can now follow the pipeline as indicated in the directed graph in Figure 2. We run the two scripts “Aggregate\_PSMs\_to\_Sites.Rmd” and “Process\_proteinGroups.Rmd” to prepare the input for the final script “Normalize\_MS2SiteToProtein.Rmd”. Just as the script “IM.Rmd” in the interference modeling workflow, these three scripts have their first two code blocks dedicated to 1) loading required packages and 2) specifying relevant parameters. Before running each script, make sure that the required packages are installed.

The parameters in each scrip’s parameter section are already configured to the demo. Please refer to the respective comments above the lines of code in order to get more insight into each parameter. If specified incorrectly, the program might produce errors down the line. Everything outside of the parameter code blocks does not need to be changed by the user. If a parameter is described as “Optional”, the parameter is not required for successfully running the program, as some steps in the workflow can skipped. Set optional parameters to their default value to skip corresponding code blocks. The default values of optional parameters are described in the comments of the code.

Like in the interference modeling workflow, each code block performs a specific task in the respective script and often produces intermediate output (visual and/or textual) of interest. We hope that the comments in the code provide the necessary understanding of what each code section is doing.

Please note that both scripts “Aggregate\_PSMs\_to\_Sites.Rmd” and “Process\_proteinGroups.Rmd” contain some parameters and/or optional code blocks that allow for more stringent filtering of features based on defined thresholds of score, intensity and PSM-wise PPF (Precursor Purity Fraction) values. Currently, the parameters in each script are set to filter as permissive as possible. Generally, we advise adjustment of those filter parameters in order to get rid of features with subpar data quality.

**Output**

The final script “Normalize\_MS2SiteToProtein.Rmd” produces an output table named “SiteToProtein\_normalized\_aggregated\_Acetyl (K)Sites.txt” that is stored in the “Results” folder. Please note that this table will only list sites that could be normalized to corresponding protein level (i.e. unmodified peptides of the same proteins), which naturally requires independent quantification on both site and protein level. Hence, some sites might have been removed in the process.

The output table contains multiple additional intensity columns, which are already normalized between the samples and therefore need no additional normalization. These are: Site intensities (no suffix), interference-adjusted site intensities (suffix *“\_\_IFadjust”*)*,* underlying protein intensities (suffix *“\_\_underlyingProtein”*), interference-adjusted underlying protein intensities (suffix*“\_\_underlyingProtein\_IFadjust”*)*,* site-to-protein normalized abundances that are likely biased by varying degrees of ratio compression in individual site and protein pairs (suffix*“\_\_siteToProtein”*)*,* and finally interference-adjusted site-to-protein normalized abundances that mitigate this aforementioned bias (suffix*“\_\_siteToProtein\_IFadjust”*)*.*

Please note that “interference-adjusted” here does not imply “interference-corrected”. Instead it means that the interference levels in each individual site and protein pair have been equalized to reach the same level such that subsequent ratio-building is unbiased (i.e. not biased by different levels of reporter ion interference). For more information, please refer to our publication: <https://doi.org/10.1016/j.mcpro.2023.100694>

If a batch vector was specified in the parameter section of “Normalize\_MS2SiteToProtein.Rmd”, the output table contains additional columns for all intensity types that are batch corrected via the comBat algorithm (additional suffix *“\_\_batchCorr*”). Further, the table contains ANOVA p-values and other metrics of interest like the column “*max\_abs\_log2\_FC\_siteToProtein\_IFadjust*”, which gives a measure of the maximum effect size/amplitude of change in the PTM modification rate of one group versus all other.

The scripts “Aggregate\_PSMs\_to\_Sites.Rmd” and “Process\_proteinGroups.Rmd” produce intermediate output tables that are stored in the “Results” folder. These tables might be relevant in their own way since they still contain the full list of sites and proteins prior to filtering based on independent measurement on both site and protein level, including columns for normalized as well as interference-corrected intensities.

**Additional Comments**

For the sake of simplicity, this demo only covered the normalization of MS2-quantified site intensities to MS3-quantified protein intensities. On MS3-quantified protein level, we make the (simplifying) assumption that no ratio compression effects are present (i.e. EIL = 0). However, the workflow also supports normalization to MS2-quantified protein intensities where EIL is assumed to be  ≥ 0. This requires an extra round of interference modeling for PSMs of MS2-quantified unmodified peptides (i.e. “proteome) to ultimately estimate the degree of interference in MS2-quantified proteins, in addition to MS2-quantified sites (see Figure 4 below). Other than this, the rest of the workflow stays the same. In the diagram below, the full workflow to perform normalization of MS2-quantified site abundances to MS2-quantified protein abundances is depicted:

Diagram

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***Figure 4 -*** *Overview of the site-to-protein normalization workflow when normalizing MS2-quantified sites to MS2-quantified proteins. In blue: MaxQuant output; in green: R-scripts; in orange: Intermediate or final data output.*

The demo user can try this workflow for themselves – half of the workflow (i.e. the left side in Figure 4) has already been performed as part of this demo. All the required input data is available on PRIDE (identifier PXD040449) among the search results named “MaxQuant\_SiteToProteinNorm\_txt.zip”. The required raw files from measurements of unmodified peptides (i.e. “proteome”) via MS2-based quantification are named:

20201030\_QExHFX1\_RSLC1\_Madern\_Hartl\_UW\_MFPL\_\_complexity\_P2

20201030\_QExHFX1\_RSLC1\_Madern\_Hartl\_UW\_MFPL\_\_complexity\_P5

They are also available on PRIDE (identifier PXD040449). Please note that the workflow shown in Figure 4 will require a second instance of the script “IM.Rmd”, as well as the corresponding PSM level output. Therefore, we recommend setting up and run the “protein-half” of this pipeline (i.e. the right half in Figure 4) in a different directory to where the other half is located.