Application of Parallel Reaction Monitoring in ^{15}N labeled Samples for Quantification

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Abstract

Accurate relative quantification is critical in proteomic studies. The incorporation of stable isotope ^{15}N to plant-expressed proteins *in vivo* is a powerful tool for accurate quantification with a major advantage of reducing preparative and analytical variabilities. However, ^{15}N labeling quantification has several challenges. Less identifications are often observed in the heavy labeled samples because of incomplete labeling, resulting in missing values in reciprocal labeling experiments. Inaccurate quantification can happen when there is contamination from co-eluting peptides or chemical noise in the MS^1 survey scan. These drawbacks in quantification can be more pronounced in less abundant but biologically interesting proteins, which often have very few identified peptides. Here we demonstrate the application of parallel reaction monitoring (PRM) to ^{15}N labeled samples on a high resolution, high mass accuracy Orbitrap mass spectrometer to achieve reliable quantification even of low abundance proteins in samples.

1. 1 Introduction

Relative quantitative comparisons between biological samples are critical in proteomic studies to uncover key differences of interest. The incorporation of stable isotope ^{15}N to labeled proteins in plants is one powerful strategy that can be used to achieve such information. In such a quantitative experiment, one sample contains the most common light isotope (^{14}N), and the other is labeled with a stable heavy isotope (^{15}N) in the form of nitrogen salts, such as $^{15}NH_4{}^{15}NO_3$, $K^{15}NO_3$, or $^{15}NH_4Cl$. After mixing these samples, relative quantitative information is achieved by comparing the intensity between the light and heavy isotopic peptide peaks. Because the samples can be mixed at the beginning of sample processing, ^{15}N metabolic labeling significantly reduces preparative and analytical variabilities and allows the relative quantification of thousands of proteins simultaneously.

In ^{15}N labeling experiments, a reciprocal labeling experiment is necessary (Arsova et al., 2012; Skirycz et al., 2011; Wang et al., 2002). In this strategy, reverse labeling is implemented by switching which sample is metabolically labeled in a biological replicate. The reciprocal labeling experimental design eliminates experimental bias, including labeling effects and quantitation errors (Arsova et al., 2012; Shrestha et al., 2021). Proteins that show consistent up or down-

regulation between these reciprocal experiments are considered reliable results for differentially expressed/regulated proteins.

Despite its great potential, ^{15}N labeling has several challenges. Incomplete incorporation of the heavy isotope is often observed, resulting in satellite isotope patterns and less identification of the ^{15}N heavy-labeled peptides (Minkoff et al., 2014; Shrestha et al., 2021). This incomplete labeling leads to fewer numbers of proteins being identified in heavy-labeled samples compared to light-labeled samples, thus causing missing values in quantification in reciprocal labeling experiments. A second challenge comes from co-eluting contaminating peptides or chemical noise which leads to inaccurate quantification using MS^1 intensities. In highly complex samples, even with high-resolution data acquisition, co-eluting peptides or chemical noise still exist and skew quantification. These drawbacks are more pronounced in lower abundance proteins where very few peptides are typically identified and quantified. While these challenges are not unique to ^{15}N metabolic labeling, solutions are desired to improve the quantification quality.

Here we present utilizing Parallel Reaction Monitoring (PRM) (Peterson et al., 2012) in ^{15}N labeling experiments to achieve more accurate quantification results (Bi et al., 2021; Park et al., 2019). In the PRM method, targeted peptide ions are mass filtered, usually using a quadrupole, and subsequently fragmented, all the yielded fragment ions are then detected, in our case using a high-resolution Orbitrap mass analyzer (Figure 1). As it is targeted quantification, all peptides of interest are fragmented in reciprocal labeled replicates. Also, because the quantification is done using MS^2 fragment ions measured in a high resolution, high accuracy instrument, signals from co-eluting peptides or chemical noise can be easily eliminated, leading to more accurate quantification. In this method, we first show examples of the challenges in ^{15}N quantification using non-targeted MS^1 of protein SAP18, and then we show step-by-step how to set up PRM for the light ^{14}N and heavy ^{15}N target peptides using Skyline as well as how to set up PRM acquisition methods on an Orbitrap Q Exactive HF. We show how to use Skyline to analyze the data. We then present examples of how PRM quantifies housekeeping protein TUBULIN and provides unambiguous quantification of SAP18 protein. This method is applicable to protein-level quantifications in ^{15}N labeling samples and can also be applied to post-translational modified peptides with slight modifications (Bi et al., 2021; Park et al., 2019).

2.1 Materials and equipment.

Data from two batches of samples are presented. In the first set, materials and data acquisition were detailed in (Bi et al., 2021), in which three biological samples were generated: one forward sample F1 (^{14}N Col/^{15}N *acinus-2 pinin-1*) and two reverse samples R1 and R2 (^{14}N *acinus-2 pinin-1*/^{15}N Col). MS data were acquired on a Q-Exactive HF instrument with Easy-spray column ES803 (50 cm x 75 µm ID, PepMap RSLC C18, 2 µm, ThermoFisher) and analyzed using Protein Prospector(Chalkley et al., 2005) and Skyline(Schilling et al., 2012).

The second batch of samples was generated using the same seedling growth conditions as described in (Bi et al., 2021). Detailed descriptions can be found in supplementary data. Briefly, ^{14}N labeled Col and ^{15}N labeled *acinus-2 pinin-1* were mixed about 1:1 ratio and data-dependent acquisition (DDA) and targeted quantification (PRM) data were acquired on Q-Exacive HF

instrument with trapping column Acclaim PepMap 100 (75 uM x 2cm, C18, 3 µm, Thermo Fisher), then separated using analytical column Acclaim PepMap RSLC (75um x 25cm, C18, 2 µm, Thermo Fisher).

The following items are required for data analysis in this method.

1. Skyline software (free download from http:://skyline.ms)
2. (Optional) Microsoft office, including Excel
3. (Optional) R studio
4. MS Thermo Raw FileReader package.

3. Method

3.1 Generate the target peptides list.

To target proteins of interest (POI) for PRM analysis, it is necessary to get peptide information for those targets, including peptide sequence, *m/z* (mass to charge ratio), and retention time. This information can be retrieved either from prior ^{15}N DDA data analysis or from publicly available databases. If from the latter, then a wider monitoring window is required since the retention time can shift due to different columns or gradients.

As peptides serve as a proxy to proteins, selecting target peptides that enable the best results is critical to yield the most accurate ratio that is truly representative of POI. Criteria for compatible target peptides:

- Peptide length: 6-25 amino acids long.
- Missed cleavages: peptides should have no missed cleavages.
- Not expected to be modified: Peptides containing methionines or tryptophans are generally not a good choice as they could become partly oxidized, splitting the signal between multiple masses.
- Intensity: Peptides from the same protein have different ionization efficiencies, so choosing a peptide that gave a strong signal when previously analyzed will give better quantification accuracy.
- Note: if there are multiple peptides to choose from, then following the criteria above will generate the optimal results. If few peptides have been previously identified, then the choice may be limited.

3.2. Generate the Skyline template and export the information.

After creating a list of target peptides, the next step is to create a Skyline template for them. This will serve as a method to calculate masses of the light and heavy versions of the peptides and will be used downstream to import and quantify peptide ratios once PRM data is acquired.

3.2.1 Organize the peptide sequence and protein name into a two-column excel file:
Table 1: Example of Skyline peptide list.

Peptide Sequence	**Protein Name**
EVGETMAYPNR	SAP18
LSFAFVYPNNK	SAP18

LAVNLIPFPR	TUB2
YTGDSDLQLER	TUB2

3.2.2 Import peptides into Skyline:
There are multiple ways to generate peptide templates in Skyline: a. manually; b. using R script; c. using a library.
Manual template generation involves two steps: 1) generate the light version; 2) modify the sequence to the heavy version with heavy isotope modification. Once modified, Skyline will automatically include the light version.

- To generate the light version, the most straightforward way is to copy the peptide sequence and protein name from an excel sheet as listed in table 1 and paste it into the peptides menu in Skyline. The path to insert peptides into Skyline:
  Skyline > Edit > Insert > Peptides... > copy excel into skyline box ->insert.
  Now the template containing the light version is generated.
- Modify the sequence to the heavy version with heavy isotope modification. Right-click on the peptide and click on edit modification. It is important to add ^{15}N isotope heavy modification to Skyline. Detailed instructions are described in Supplemental Figure 1. Once all amino acids in the sequence have been modified with a heavy isotope, then the heavy version of the peptide is generated. Skyline will automatically include the light version.

Generate template using an R script: A faster way to insert target peptide pairs is by utilizing a homemade R script (available upon request) that can automatically generate heavy peptide sequences recognizable by Skyline. The sequence of the peptide is converted to a heavy version using the formula as listed in Table 2. The input file for the R script is the same as Table 1, and the output after running the script is a CSV file containing the heavy peptide sequences (Table 3). Insert the peptide sequence and protein name to Skyline as described above and Skyline will generate both light and heavy peptide templates.

Table 2: Light ^{14}N to heavy ^{15}N amino acid conversion for 20 amino acids.

Amino Acid	**Conversion**
A,D,E,F,G,I,L,M,P,S,T,V,Y	[+1]
K,N,Q,W	[+2]
H	[+3]
R	[+4]
C + Carbamidomethylation	[+58]
M + oxidation	[+17]

Table 3: Output from the R script that converts peptide sequence to heavy peptide sequence recognizable by Skyline.

Peptide Sequence	Protein Name
E[+1]V[+1]G[+1]E[+1]T[+1]M[+1]A[+1]Y[+1]P[+1]N[+2]R[+4]	SAP18
L[+1]S[+1]F[+1]A[+1]F[+1]V[+1]Y[+1]P[+1]N[+2]N[+2]K[+2]	SAP18
L[+1]A[+1]V[+1]N[+2]L[+1]I[+1]P[+1]F[+1]P[+1]R[+4]	TUB2
Y[+1]T[+1]G[+1]D[+1]S[+1]D[+1]L[+1]Q[+2]L[+1]E[+1]R[+4]	TUB2

Generate the template using a library. Templates can be generated using the library function. Identified peptide information is first exported from search engine results and imported to Skyline. Detailed instructions can be found in Skyline tutorials (https://skyline.ms/_webdav/home/software/Skyline/%40files/tutorials/MethodEdit-2_5.pdf?listing=html) and will not be covered here. The advantage of using a library is that it allows the generation of templates for hundreds or more peptides and it can contain a retention time which potentially can be used for data filtering in the next steps. The downside of using the library is that the light and heavy peptides are input as separate entries, creating potential problems for data analysis unless users manually curate the template using steps as mentioned above.

3.2.3 Export list containing mass information of paired peptides from Skyline
Once all the appropriate target peptides are represented in the Skyline session, the light and heavy lists are exported to help generate the spreadsheet needed for the PRM acquisition method in the Orbitrap mass spectrometer.
The path to export the skyline list is:
Skyline > File > Export > Report.

Export a custom report that only contains peptide sequence, precursor charge, protein name, and precursor m/z.
To create a custom report, navigate:
Edit List > Add.. > Check Peptide Sequence, Precursor Charge, Protein Name, and Precursor Mz

3.3 Add retention time and create PRM template (inclusion list)

3.3.1 Add retention time window
A critical component of the PRM assay is the inclusion of a retention time window. From either a previous data-dependent acquisition MS run or from a public database, copy and paste the recorded retention time for those target peptides into the report file generated by Skyline with a header labeled as the retention time. Then add a monitoring window that specifically corresponds to the precursor survey retention time window. A narrow window (±5 min or less) can be chosen if the same chromatographic conditions in DDA are to be used for targeted quantification. Otherwise, a wider window is recommended to make certain that the target peptides are monitored. Notably, the numbers of the targets, cycle time, and the data points

across the elution profile for the PRM method are detailed in (Gallien et al., 2014; Peterson et al., 2012; Rauniyar, 2015).

3.3.2 Make a PRM template
This adapted Skyline report now has all the information needed to produce the form needed by the Orbitrap instrument to target these peptides. The next step is to create a csv file with the following headers, which can be imported into the PRM template via global list/inclusion list.

Table: PRM template as an inclusion list.

Mass [m/z]	Formula [M]	Formula type	Species	CS [z]	Polarity	Start [min]	End [min]	(N) CE	(N)CE type	MS X ID	Comment
650.3402				2	Positive	87.35	107.35	27	NCE		LSFAFVYPNNK
657.3195				2	Positive	87.35	107.35	27	NCE		L[+1]S[+1]F[+1]A[+1]F[+1]V[+1]Y[+1]P[+1]N[+2]N[+2]K[+2]
633.7928				2	Positive	36.43	56.43	27	NCE		EVGETMAYPNR
641.2706				2	Positive	36.43	56.43	27	NCE		E[+1]V[+1]G[+1]E[+1]T[+1]M[+1]A[+1]Y[+1]P[+1]N[+2]R[+4]

For a Q-Exactive HF, we recommend the “(N)CE” column should be “27” for all rows; the same as used in the DDA method. The first column numbers are generated from Skyline. Only the mass, polarity, retention time window, and collision energy information are required in the inclusion list.

3.4 **Create PRM method on Orbitrap Analyzer**.
The PRM method is set up using PRM in Q Exactive HF Orbitrap MS. Recommended parameters include MS^2 resolution: 60,000, AGC target: 2e5, Maxim injection time IT: 200ms, Isolation window: 1.4 m/z, (N)CE: 27. Method runtime and chromatography conditions should be the same as used in DDA. Note: Max injection time can be varied depending on the abundance of peptides and the number of peptides targeted at a particular retention time.

3.5 Import to Skyline and analyze the data.

Once the targeted data is acquired, it can be analyzed in the software Skyline.
With the previously created peptide template in 3.2.2, we must first adjust transition settings so the raw data can be properly imported into Skyline, see supplemental figure 2. Raw data can be imported directly into Skyline by following the path:
File > Import > Results >...
Once imported, quantification between each target peptide light and heavy can be analyzed.

4. Results
Two common challenges in ^{15}N quantification: missing values, contamination from co-eluting peptides.

Missing values in reciprocal labeled experiments is a common issue. This problem is pronounced in low abundance proteins, which are often the most biologically interesting. For instance, in our experiments when we compared global proteomes between wild-type and *acinus-2 pinin-1* mutants, we were interested in quantifying SAP18 protein abundance. SAP18 is of relatively lower abundance and is hypothesized to interact with ACINUS, PININ and SR45 proteins (Bi et al., 2021). We performed three ^{15}N metabolic labeling experiments, including one forward sample F1 (^{14}N Col/^{15}N *acinus-2 pinin-1*) and two reverse samples R1 and R2 (^{14}N *acinus-2 pinin-1*/^{15}N Col) experiments. We were only able to identify and quantify SAP18 in two experiments (F1, R1), but not in the R2 experiment (Figure 2, A and B). In F1, we observed SAP18 had a dramatic reduction in the *acinus-2 pinin-1* double mutant. Four peptides were identified from Col, but not the double mutant, with three peptides showing consistent reductions in the mutant, such as the peptide shown in figure 2C, while one peptide quantification shows an outlier result (A, D). We reasoned that the quantification ratio 0.386 of peptide "EVGETMAYPNR" is an outlier result because the matching peaks of the ^{15}N peptide were contaminated with a co-eluting ^{14}N labeled peptide. We reason this is a co-eluting ^{14}N labeled peptide based on the isotope pattern and that the M-1 peak is missing (labeled with black arrow) and given the labeling efficiency is 97%.

In contrast, in the reverse labeling experiment R1, in which Col is ^{15}N labeled, SAP18 was only identified with a single peptide from Col, but quantification based on this peptide is wrong because the ^{14}N peak isotope cluster is incorrect (Figure 2E) and the ^{14}N monoisotopic peak is likely contaminated with chemical noise. In the reverse labeling experiment R2, no peptides were identified from SAP18 in both Col and *acinus-2 pinin-1,* hence no quantification is possible (Figure 2A and 2B).

Parallel Reaction Monitoring (PRM) on ^{15}N metabolic samples

Targeted quantification using MS^2 can address the challenges illustrated above. We have previously employed PRM to phosphopeptides in label-free samples (Ni et al., 2017) and phosphopeptides in ^{15}N samples (Bi et al., 2021; Park et al., 2019). Targeted quantification of ^{15}N samples has also been implemented by the Sussman lab using Selective Monitoring Reaction using a low-resolution triple quadrupole mass spectrometer (Minkoff et al., 2015)**.** Due to the high complexity of the samples, because all proteins were extracted, PRM on a high resolution, high accuracy Orbitrap mass spectrometer should provide more accurate results. TUBLIN2 proteins (Figure 3), as well as 42 different peptide pairs (light and heavy) from 30 relatively abundant proteins in *Arabidopsis* (Supplemental Figure 3), were set up for PRM targeted quantification. From this data, we have observed that: 1) ^{14}N and ^{15}N peptides almost co-elute, with ^{15}N eluting about 2-4 sec earlier in a 2-hour gradient (supplemental Figure 3). 2) The light and heavy pair of peptides produce similar fragmentation patterns, illustrated in Figure 3 and supplemental Figure 3. Each colored line represents the same fragment ion between the light and heavy pairs. 3) Peptides from the same protein give consistent quantification in PRM.

We set up targeted quantifications for the peptides "EVGETMAYPNR" and "LSFAFVYPNNK" from SAP18 as described in the method section. The PRM quantification on "EVGETMAYPNR"

peptides showed a consistent reduction of this peptide in the *acinus-2 pinin* double mutant in all three replicates, confirming the ^{15}N peak quantification in MS^1 spectra was indeed incorrect due to co-eluting peptide contamination (Figure 4A, Figure 2D). For the peptide "LSFAFVYPNNK", the PRM quantification also showed a consistent reduction of signal in the double mutant (Figure 4B). More importantly, both peptides were quantified in the R2 sample, which was not quantified in the untargeted analysis. We also performed targeted quantification of a third peptide "QGGGRPLPPPPR" (Bi et al., 2021). Altogether, we concluded SAP18 protein levels were dramatically reduced to 2.7% of WT levels after normalization, indicating that the stability of the other members of the ASAP and PSAP complexes is dependent on ACINUS and PININ in Arabidopsis.

5. Discussion

Targeted acquisition of ^{15}N samples has previously been reported (Hart-Smith et al., 2017), where an inclusion list for POI was added to data acquisition, such that more proteins were repeatedly identified and quantified across multiple runs. However, the quantification was done at the MS^1 level, so although this addresses the missing value problem, the quantification accuracy is still affected by contaminating co-eluting peaks.

We demonstrated using PRM targeted quantification we were able to quantify SAP18 with consistent quantification results in all replicates. SAP18 is not only of low abundance but it is also relatively small (18 KDa) so it produces few peptides, both of which contribute to the challenge of quantifying this protein. In the same assay, we also performed a targeted assay on peptides spanning regions before and after the T-DNA insertion of ACINUS and PININ (Bi et al., 2021). The PRM results demonstrated ACINUS's N-terminus (before the T-DNA insertion) was expressed in the mutant at about 20% WT level, but the C-terminus (after the T-DNA insertion) was not detectable. In contrast, using the PRM method, we showed both the N- and C-termini of PININ (before and after the T-DNA insertion) were detectable in Col but were not detectable in the double mutant. Notably, the PININ protein was of relatively low abundance and was not detected in DDA data. We were able to set up PRM for PININ using the retention time and peptide information obtained from previous immunoprecipitation results (Bi et al., 2021).

Combining PRM and ^{15}N labeling has additional advantages. As shown in Figure 4, SAP18 is quantified using both ^{14}N labeled and ^{15}N labeled peptides that co-elute, and as MS^2 of these pairs produce the same fragments but with different masses, the false-discovery rate of these peptides are minimal, thus the quantification of the proteins is even more reliable than using label-free samples.

Data Availability Statement

The dataset for this study has been deposited in PRIDE.

Author Contributions

A.V.R., R.S., and S.-L.X. designed the experiments, and A.V.R and R.S. performed the experiment, A.V.R., R.S., and S.-L.X. analyzed the data sets and generated figures. P.R.B. and

R.J.C. provided technical support for Protein Prospector and revised the manuscript. A.V.R., R.S., and S.-L.X. wrote the manuscript.

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Conflict of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

Supplemental Material
The supplemental figure 1,2,3 and the supplemental method are provided.

Figure Legends

Figure 1: Schematic view of combining Parallel Reaction Monitoring to ^{15}N metabolic labeling quantification workflow to get unambiguous quantification results. Light (^{14}N) (colored in red) or heavy (^{15}N) labeled peptide (colored in blue) ions are isolated within small m/z windows in the first quadrupole (Q1) and then fragmented using higher-energy collision dissociation (HCD) in the second quadrupole. The MS^2 fragments are separated and detected by Orbitrap. Data is imported to Skyline and the area under the curve (AUC) of the fragments are integrated for quantification.

Figure 2: Quantification challenges in ^{15}N metabolic labeling samples. (A and B) Boxplots of protein ratio measurements from reciprocal ^{15}N labeling experiments: one forward sample F1 (^{14}N Col/^{15}N *acinus-2 pinin-1*) and two reverse samples R1 and R2 (^{14}N *acinus-2 pinin-1*/^{15}N Col). Each dot represents the ratio for a single peptide identified to each protein. TUBULIN is quantified in all three experiments based on many peptides, while SAP18 is quantified in two of three experiments (F1, R1), with inaccurate quantification in R1, and no quantification in R2. (C) Spectrum showing correct quantification of ^{14}N and ^{15}N pair for peptide “EVGETM(oxidation)AYPNR” from SAP18 protein in the F1 sample. (D) Quantification for peptide “EVGETMAYPNR” is inaccurate because the ^{15}N matching peak is contaminated with a co-eluting peptide in F1 sample. (E). Quantification for peptide “LSFAFVYPNNK” is wrong as the matching ^{14}N peak is contaminated with overlapping peaks. Blue and green arrows point to ^{14}N and ^{15}N monoisotopic peaks, respectively.

Figure 3: PRM quantification on TUBULIN2. (A and B) Two peptides from TUB2 were quantified in three samples. The six most abundant fragment ions are annotated with mass in the right legend with the order from most to the least abundant. The light and heavy peptides generate the same pattern of fragment ions.

Figure 4: Targeted quantification using PRM on SAP18 proteins provides unambiguous quantification results. The targeted quantifications in three samples gave consistent results

showing SAP18 was dramatically reduced in *acinus-2 pinin-1* mutant. The light and heavy peptides gave a similar pattern of fragmentation. The peptides are quantified using area under the curve (AUC).

Supplemental Figure 1: Example of steps for adding heavy isotope modification.

Supplemental Figure 2: Example of setting up transition setting.

Supplemental Figure 3: PRM quantification on multiple proteins. 42 pairs of peptides (^{14}N and ^{15}N peptides) are quantified using the ^{14}N Col/^{15}N *acinus-2 pinin-1* sample and 9 pairs are displayed. The top chromatograph represents ^{14}N light fragment ions, and the bottom represents the ^{15}N heavy fragment ions. The apex of the heavy peak is centered with the dashed line. The light and heavy peptide almost co-elute, with the heavy version elutes about 2-4 seconds earlier than the light version.

Table 1: Example of Skyline peptide list.

Table 2: Light ^{14}N to heavy ^{15}N amino acid conversion for 20 amino acids.

Table 3: Output from the R script that converts peptide sequence to heavy peptide sequence recognizable by Skyline.

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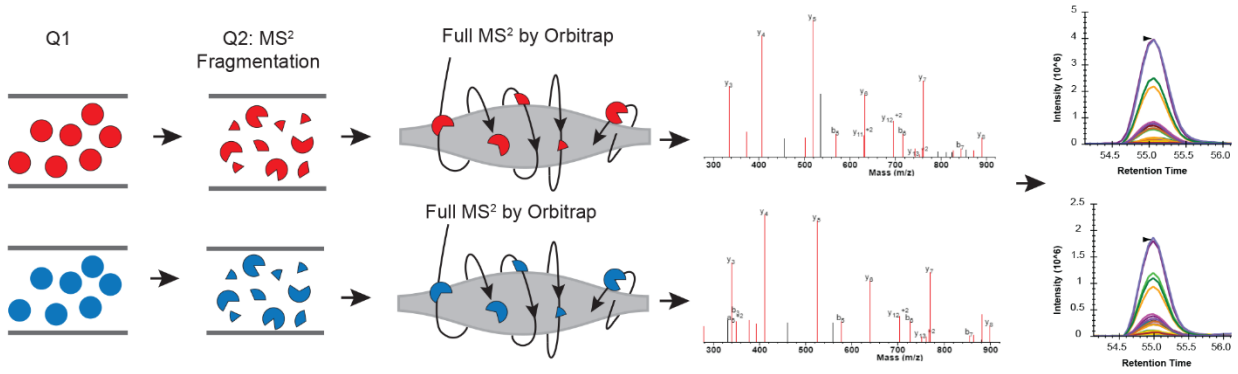
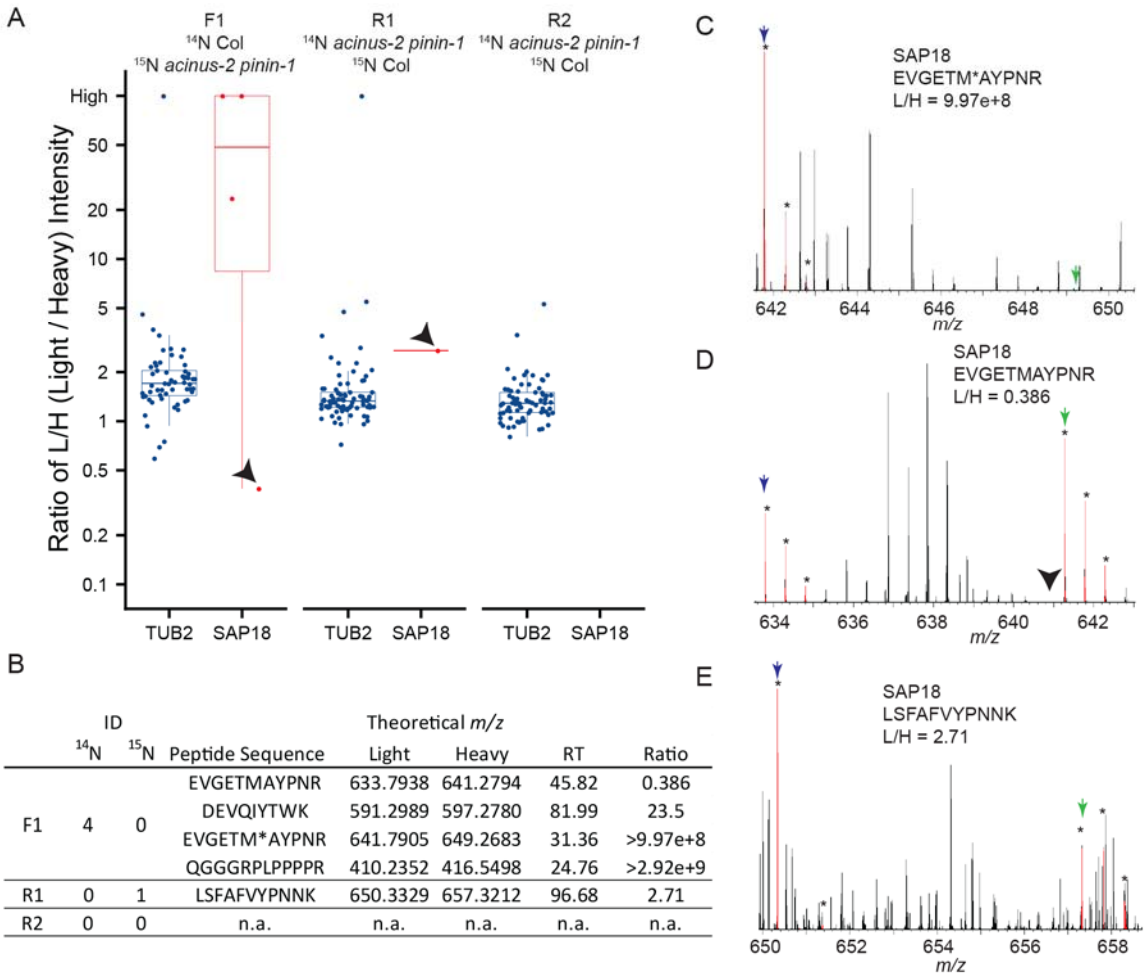


Figure 1: Schematic view of combining Parallel Reaction Monitoring to ^{15}N metabolic labeling quantification workflow to get unambiguous quantification results. Light (^{14}N) (colored in red) or heavy (^{15}N) labeled peptide (colored in blue) ions are isolated within small m/z windows in the first quadrupole (Q1) and then fragmented using higher-energy collision dissociation (HCD) in the second quadrupole. The MS^2 fragments are separated and detected by Orbitrap. Data is imported to Skyline and the area under the curve (AUC) of the fragments are integrated for quantification.



B

ID				Theoretical *m/z*			
	^{14}N	^{15}N	Peptide Sequence	Light	Heavy	RT	Ratio
F1	4	0	EVGETMAYPNR	633.7938	641.2794	45.82	0.386
			DEVQIYTWK	591.2989	597.2780	81.99	23.5
			EVGETM*AYPNR	641.7905	649.2683	31.36	>9.97e+8
			QGGGRPLPPPPR	410.2352	416.5498	24.76	>2.92e+9
R1	0	1	LSFAFVYPNNK	650.3329	657.3212	96.68	2.71
R2	0	0	n.a.	n.a.	n.a.	n.a.	n.a.

Figure 2: Quantification challenges in ^{15}N metabolic labeling samples. (A and B) Boxplots of protein ratio measurements from reciprocal ^{15}N labeling experiments: one forward sample F1 (^{14}N Col/^{15}N *acinus-2 pinin-1*) and two reverse samples R1 and R2 (^{14}N *acinus-2 pinin-1*/^{15}N Col). Each dot represents the ratio for a single peptide identified to each protein. TUBULIN is quantified in all three experiments based on many peptides, while SAP18 is quantified in two of three experiments (F1, R1), with inaccurate quantification in R1, and no quantification in R2. (C) Spectrum showing correct quantification of ^{14}N and ^{15}N pair for peptide "EVGETM(oxidation)AYPNR" from SAP18 protein in the F1 sample. (D) Quantification for peptide "EVGETMAYPNR" is inaccurate because the ^{15}N matching peak is contaminated with a co-eluting peptide in F1 sample. (E). Quantification for peptide "LSFAFVYPNNK" is wrong as the matching ^{14}N peak is contaminated with overlapping peaks. Blue and green arrows point to ^{14}N and ^{15}N monoisotopic peaks, respectively.

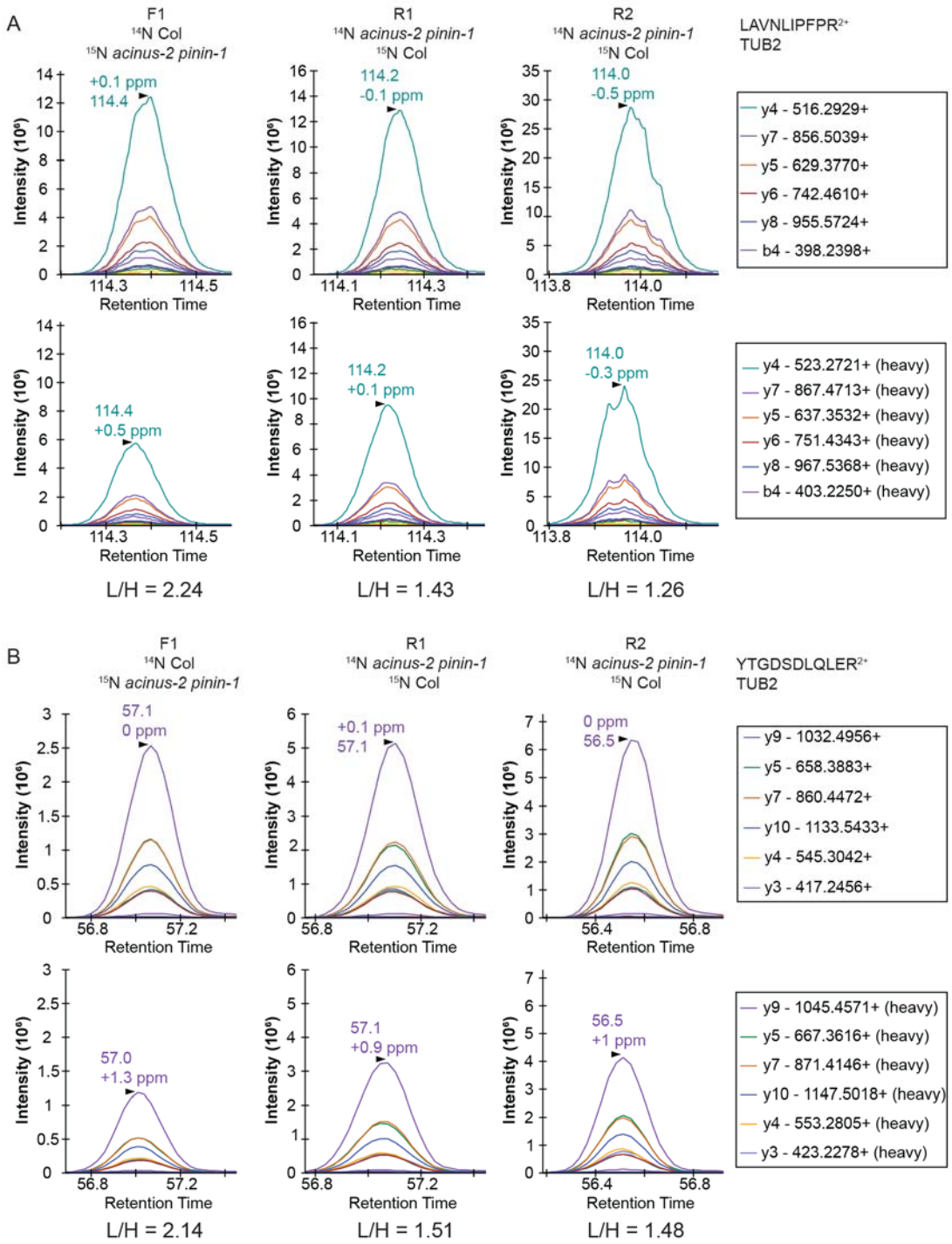


Figure 3: PRM quantification on TUBULIN2. (A and B) Two peptides from TUB2 were quantified in three samples. The six most abundant fragment ions are annotated with mass in the right legend with the order from most to the least abundant. The light and heavy peptides generate the same pattern of fragment ions.

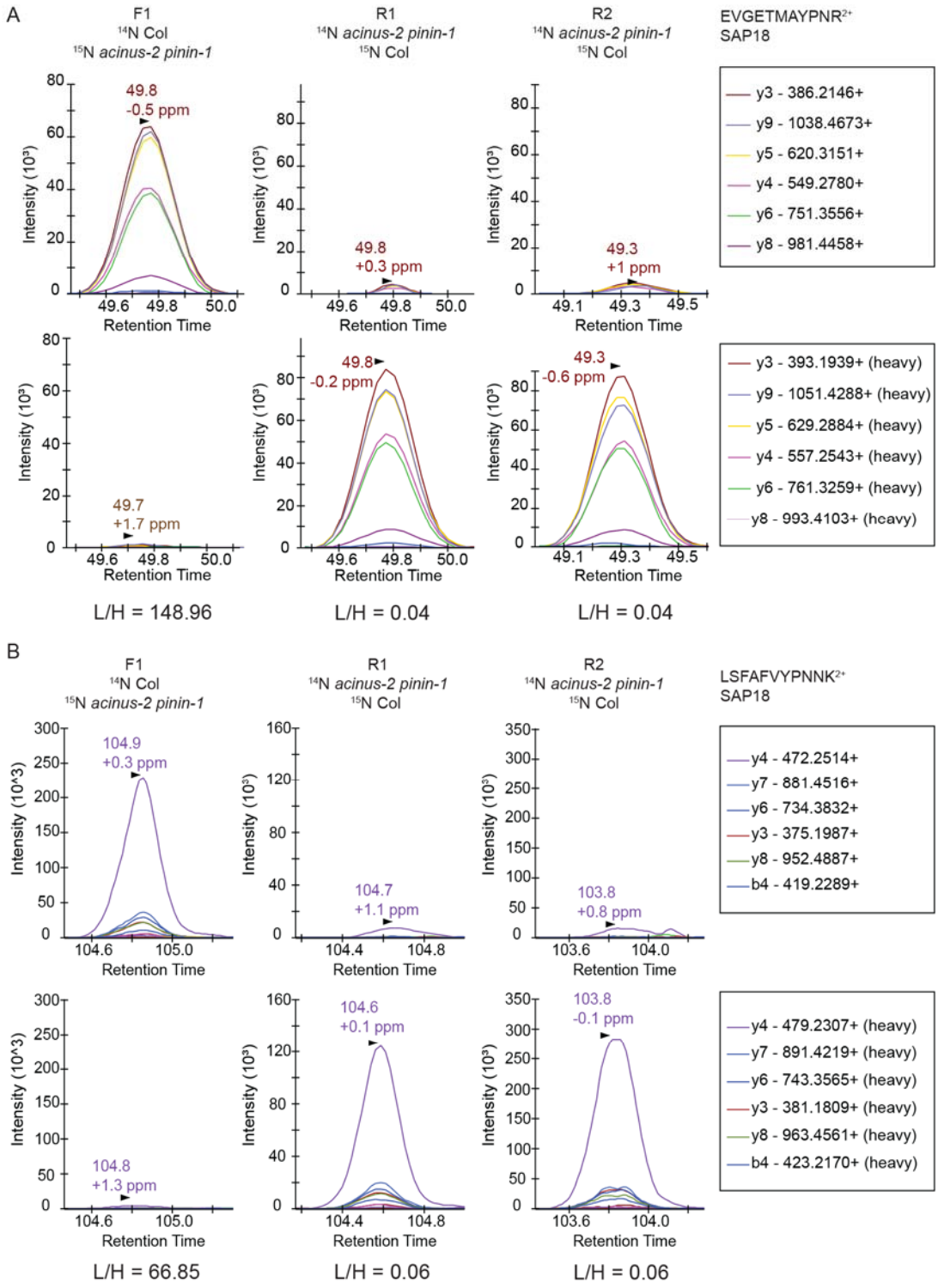
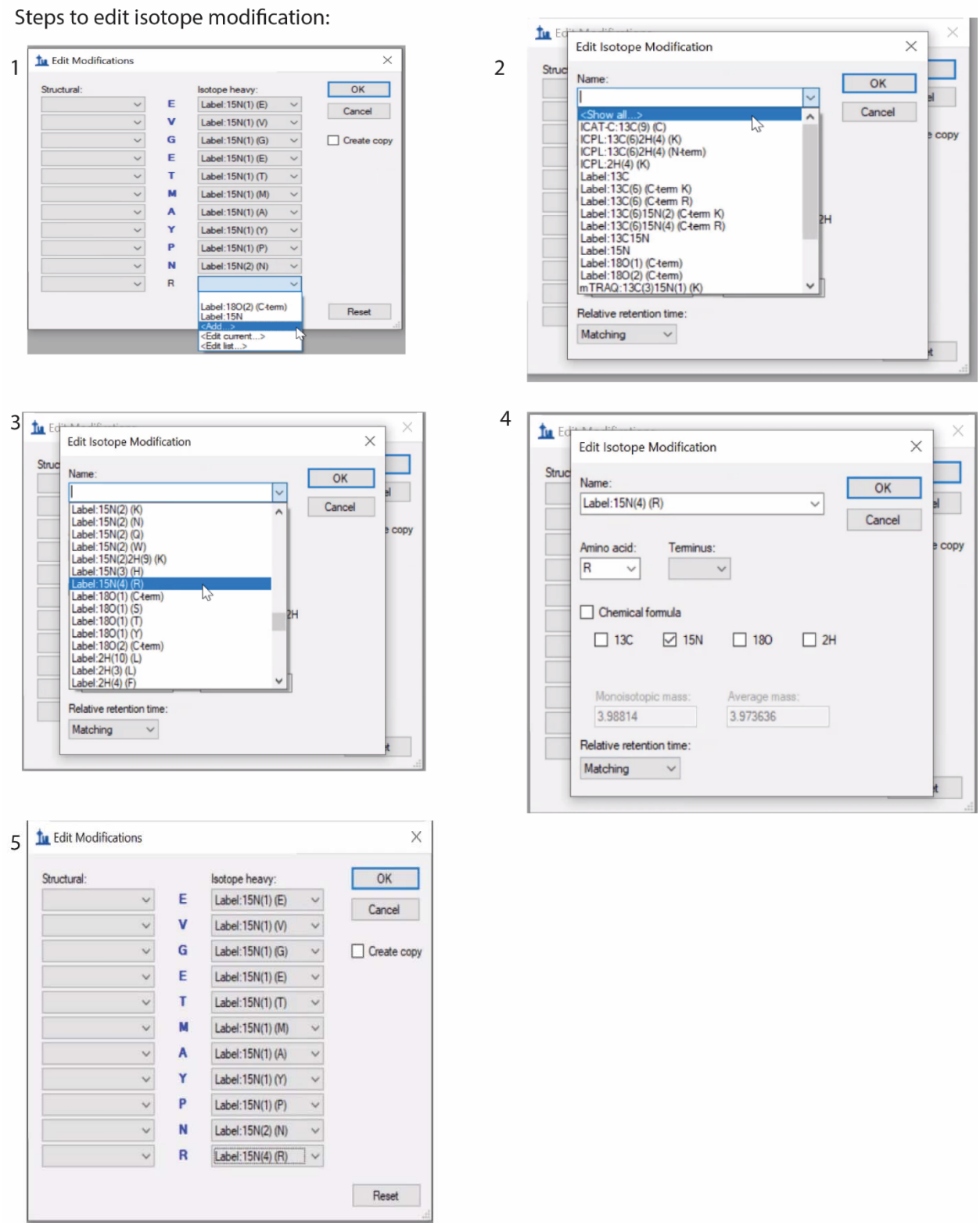
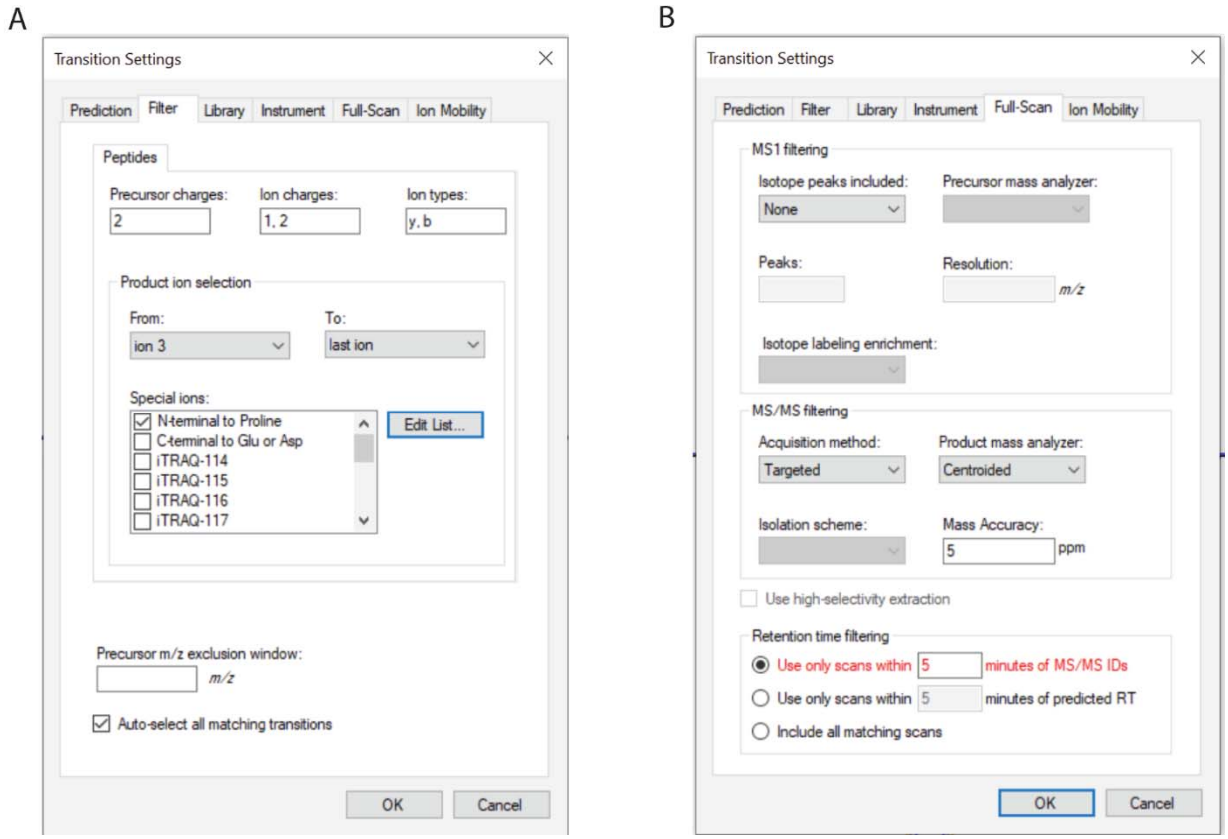


Figure 4: Targeted quantification using PRM on SAP18 proteins provides unambiguous quantification results. The targeted quantifications in three samples gave consistent results showing SAP18 was dramatically reduced in *acinus-2 pinin-1* mutant. The light and heavy peptides gave a similar pattern of fragmentation. The peptides are quantified using area under the curve (AUC).

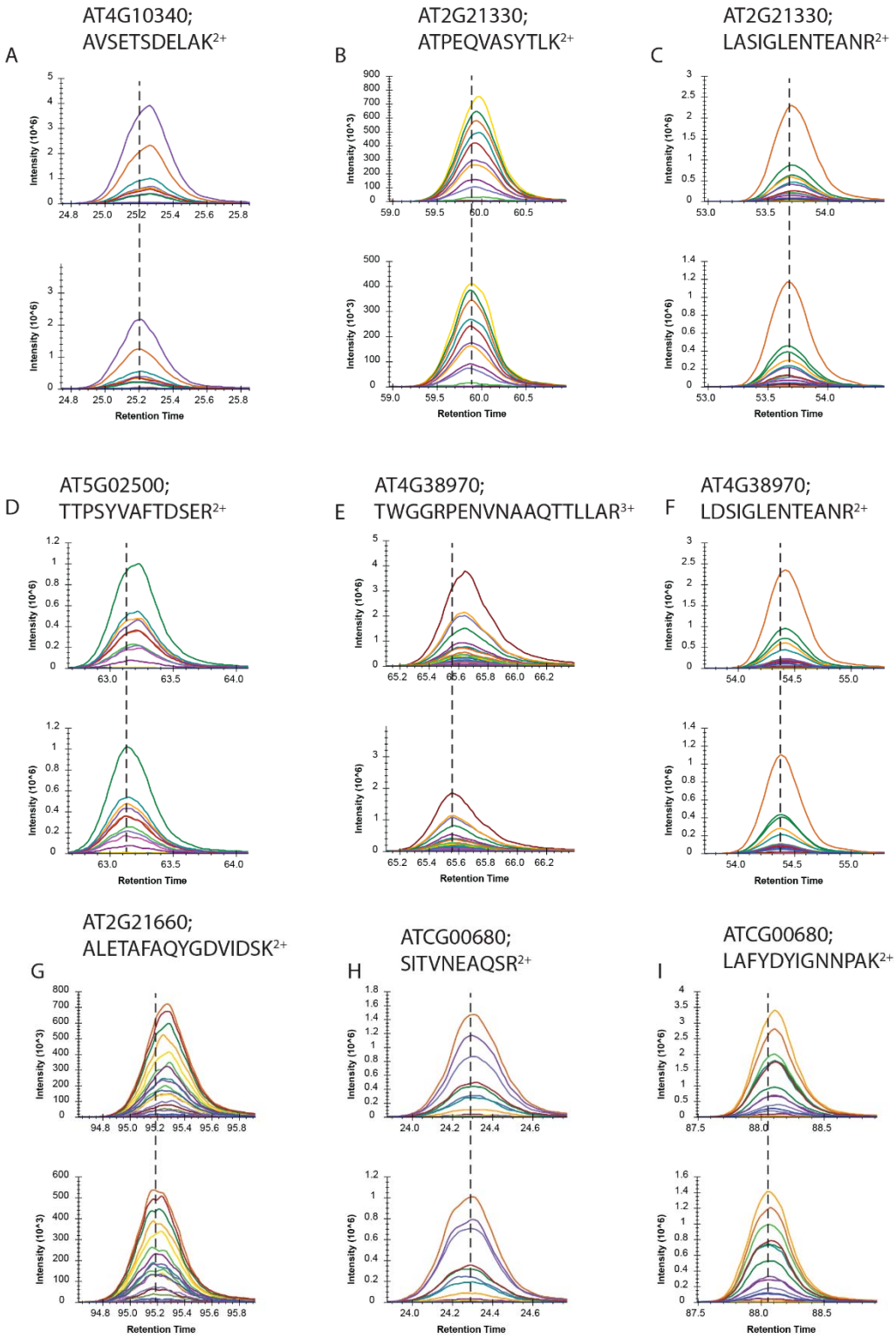
Steps to edit isotope modification:



Supplemental Figure 1: Example of steps for adding heavy isotope modification.



Supplemental Figure 2: Example of setting up transition setting.



Supplemental Figure 3: PRM quantification on multiple proteins. 42 pairs of peptides (^{14}N and ^{15}N peptides) are quantified using the ^{14}N Col/^{15}N *acinus-2 pinin-1* sample and 9 pairs are displayed, with the top chromatograph represents ^{14}N light fragment ions, and the bottom represents the ^{15}N heavy fragment ions. The apex of the heavy peak is centered with the dashed

line. The light and heavy peptide almost co-elute, with the heavy version elutes about 2-4 seconds earlier than the light version.

Supplemental Method

The WT and *acinus-2 pinin-1* plants were grown on Hoagland medium containing ^{14}N or ^{15}N (1.34 g/L Hogland's No. 2 salt mixture without nitrogen, 6 g/L Phytoblend, and 1 g/L KNO_3 or 1 g/L $K^{15}NO_3$ (Cambridge Isotope Laboratories), pH 5.8) as described as (Bi et al., 2021). Proteins were extracted from two samples (one ^{14}N-labeled Col, one ^{15}N-labeled *acinus-2 pinin-1)* followed with protocols as described (Xu et al., 2017) with slight modification. Briefly, samples were first extracted individually using SDS sample buffer (0.1 M Tris·HCl, pH 8.0; 2% (wt/vol) SDS; 20 mM EGTA; 20 mM EDTA; 1.2% (vol/vol) Triton X-100; 2x protease inhibitor), then the protein concentration of each sample was measured by BCA assay kit (Thermo Fisher), subsequently followed by mixture to have 1:1 protein concentration mixture. Then Proteins were further extracted by cold phenol extraction. Proteins were digested with trypsin and the resulting peptides were de-salted using Sep-Pak waters C18 centrifuge columns. The peptides were analyzed on a Q-Exactive HF mass spectrometer (Thermo Fisher) equipped with an Easy LC 1200 UPLC liquid chromatography system (Thermo Fisher).

Data dependent Acquisition: Peptides were first trapped using trapping column Acclaim PepMap 100 (75 uM x 2cm, nanoViper 2Pk, C18, 3 µm, 100A), then separated using analytical column Acclaim PepMap RSLC (75um x25cm, nanoViper, C18, 2 µm, 100A) (Thermo Fisher). The flow rate was 300 nL/min, and a 120-min gradient was used. Peptides were eluted by a gradient from 3 to 28% solvent B (80% (v/v) acetonitrile/0.1% (v/v) formic acid) over 100 min and from 28 to 44% solvent B over 20 min, followed by a short wash at 90% solvent B. For DDA acquisition, the precursor scan was from mass-to-charge ratio (m/z) 375 to 1600 and top 20 most intense multiply charged precursors were selected for fragmentation. Peptides were fragmented with higher-energy collision dissociation (HCD) with normalized collision energy (NCE) 27. DDA data was analyzed as described in (Shrestha et al., 2021).

PRM Targeted quantification:
The data-dependent acquisition was used first to get the peptide information from multiple proteins with peptide mass/charge (m/z), retention time, and MS2 fragments. 42 paired peptides were picked that span the whole elution profile of 130 minutes. For targeted analysis, parallel reaction monitoring (PRM) acquisition using a 10-min window (±5 min) was scheduled with an orbitrap resolution at 60,000, AGC value 2e5, and a maximum fill time of 60 ms. The isolation window for each precursor was set at 2.0 m/z unit. PRM data was processed with a 5-p.p.m. window using skyline from ^{14}N- and ^{15}N-labeled samples.

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