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MaxQuant.Live Enables Enhanced Selectivity and Identification of Peptides Modified by Endogenous SUMO and Ubiquitin

Ivo A. Hendriks,* Vyacheslav Akimov, Blagoy Blagoev, and Michael L. Nielsen*

ABSTRACT: Small ubiquitin-like modifiers (SUMO) and ubiquitin are frequent post-translational modifications of proteins that play pivotal roles in all cellular processes. We previously reported mass spectrometry-based proteomics methods that enable profiling of lysines modified by endogenous SUMO or ubiquitin in an unbiased manner, without the need for genetic engineering. Here we investigated the applicability of precursor mass filtering enabled by MaxQuant.Live to our SUMO and ubiquitin proteomics workflows, which efficiently avoided sequencing of precursors too small to be modified but otherwise indistinguishable by mass-to-charge ratio. Using precursor mass filtering, we achieved a much higher selectivity of modified peptides, ultimately resulting in up to 30% more SUMO and ubiquitin sites identified from replicate samples. Real-time exclusion of unmodified peptides by MQL resulted in 90% SUMO-modified precursor selectivity from a 25% pure sample, demonstrating great applicability for digging deeper into ubiquitin-like modifications. We adapted the precursor mass filtering strategy to the new Exploris 480 mass spectrometer, achieving comparable gains in SUMO precursor selectivity and identification rates. Collectively, precursor mass filtering via MQL significantly increased identification rates of SUMO- and ubiquitin-modified peptides from the exact same samples, without the requirement for prior knowledge or spectral libraries.

KEYWORDS: SUMO, ubiquitin, MaxQuant.Live, MQL, precursor mass filtering, targeting exclusion

INTRODUCTION

Post-translational modification (PTM) of proteins is a widespread phenomenon, and virtually all proteins are known to be modified by some of the hundreds of known PTMs.1 Among the PTMs are those caused by ubiquitin and ubiquitin-like (Ubl) modifiers, such as the small ubiquitin-like modifier (SUMO) family of modifiers. Ubiquitin and SUMO constitute a unique class of PTMs because they themselves are also proteins.3 Ubiquitin and SUMO are both covalently and reversibly conjugated via their C-terminal glycine residues to lysine residues in target proteins,4,5 play a plethora of regulatory roles, and are of critical importance in the context of health and disease.6,7 Furthering this, chemical inhibitors targeting these PTMs are being used or investigated for regulatory roles, and are of critical importance in the context of health and disease.6,7 Furthering this, chemical inhibitors targeting these PTMs are being used or investigated for combating cancer.8–10

Mass spectrometry (MS)-based proteomics has emerged as an indispensable tool to gain unbiased and systemic insight into which proteins and processes are affected by PTMs across different model systems and experimental contexts.11–14 Ubiquitin was first investigated in a global manner via enrichment of the diglycine remnant that remains covalently attached to target peptides after tryptic digest,15,16 with ~10 000 ubiquitin sites being mapped. More recently, a strategy was developed that enriches ubiquitin after Lys-C digestion, where >60 000 ubiquitin sites were identified.17

Other mass spectrometric methodology enhancements are actively being developed for ubiquitylation, including on-bead tandem mass tag (TMT) labeling and application of high-field asymmetric waveform ion mobility spectrometry (FAIMS).18 SUMO2/3, two virtually indistinguishable SUMO family members of which SUMO2 is essential and the most highly expressed,19 have been primarily investigated through MS via strategies entailing genetically engineered epitope-tagged SUMO2/3,20 with 40 000 SUMO2/3 sites profiled when combining overexpression of His-tagged SUMO2 with proteasomal inhibition.21 More recently, we developed a method that allows for systems-level analysis of endogenous and native SUMO2/3,22 with 14 000 and 2000 SUMO sites mapped from cancer cells and mouse organs, respectively, and 1500 SUMO sites identified from mouse embryonic stem cells.23 Other methods for the study of SUMO exist, including a strategy that specifically profiles endogenous SUMO1,24 and...
Figure 1. Evaluation of MaxQuant.Live for analysis of peptides modified by endogenous SUMO2/3. (A) Schematic overview of the purification strategy used to enrich SUMOylated peptides. (B) Carboxy-terminal sequence of SUMO2/3, conjugated via its terminal glycine residue to the lysine in target peptides. Arrows indicate where the specified proteolytic enzymes cleave the SUMO2/3 sequence. (C) Experimental design overview. Xcalibur (black) and MQL (red) runs without exclusion were performed first, after which data was immediately processed and used for runs where contaminants were excluded, cumulating data as more replicates were acquired. Afterward, data from all 40 runs were used to perform runs where all unmodified peptides were excluded (orange). Minimum mass was set to 1700 Da. \( n = 4 \) technical replicates for most runs, \( n = 3 \) technical replicates for unmodified exclusion runs. (D) Boxplot overview of the mass distribution of all identified peptides. Black line, median; black dashed line, average; box limits, 1st and 3rd quartile; whiskers, 5th and 95th percentile. Number of data points are indicated next to the boxplots. Labels: “Xcal”, Xcalibur; “M”, minimum precursor mass filtering; “EC”, exclude contaminants; “EU”, exclude unmodified; “N”, normal; “S”, sensitive; “U”, ultrasensitive. (E) As (D), but showing spectral quality for all SUMO-modified peptides, via Andromeda score. (F) Overview of the
an approach that indiscriminately identifies all Ubls via the diglycine remnant.25

While contemporary MS methods for the study of ubiquitin and SUMO have been very successful, there are challenges related to the sample preparation and the mass spectrometric analysis of the modified peptides. For example, peptides modified by large remnants of either ubiquitin or SUMO retain two N-termini after proteolytic digest, which can result in highly complex spectra after three-way fragmentation with higher-energy collisional dissociation (HCD).17,22 Moreover, the additional N-terminus, and in some cases internal arginine residues within the Ubl mass remnants, introduce additional charges on the peptides during positive-mode MS, reducing their mass-to-charge (m/z) ratio. Despite the increased mass of the modified peptides, the increased charge-state renders their m/z similar to that of unmodified peptides, making it difficult to distinguish the Ubl-modified peptides from unmodified peptides by m/z alone, which is the primary precursor property monitored by contemporary MS. Furthermore, while some PTMs such as phosphorylation can be enriched to exceptionally high peptide-purity levels,26 achieving similar purities for ubiquitin and SUMO analyses has proven more challenging, as both PTMs are much lower abundant and the immunoprecipitation of the PTMs does not allow stringent washing procedures compared to metal-affinity purification. For example, in order to successfully purify SUMOylated peptides and afterward be able to identify them, we previously described a strategy which entails an additional digestion step with Asp-N after SUMO2/3 immunoprecipitation, introducing nonmodified peptides into the sample (Figure 1A).23 This culminates in final sample purities of ~25%, down to a few percent from challenging samples such as mouse organs. While some unmodified peptides could be “flanking” peptides originating from the originally larger modified peptides, they can also be contaminants resulting either from nonspecific binding during immunoprecipitation or from the antibodies used. Similar challenges were encountered for site-specific ubiquitination studies, where an additional digestion with trypsin was utilized to facilitate the identification of LysC-digested ubiquitylated peptides.17,27

Rather than adjusting the sample preparation methodology, we reasoned that the relatively low sample purity could be mitigated or even circumvented during acquisition of the samples on the MS, aiming to take advantage of the larger mass of Ubl-modified peptides. To this end, we set out to compare the standard software operation of the Q-Exactive HF-X MS, mediated by Tune and Xcalibur, to the recently published MaxQuantLive (MQL) software.28 MQL is able to directly assume control of a Q-Exactive-type of instrument, with the capacity to follow and target up to 25,000 peptides in real time,28 and supports BoxCar analysis, which greatly boosts MS1-level sensitivity.27 MQL includes several other features that Xcalibur lacks, including the ability to selectively target or exclude specific peptides, and the ability to define a minimum precursor mass (Dalton, Da) rather than a minimum m/z (Thompson, Th) for precursor selection.

Here, we evaluated the performance of MQL for analysis of ubiquitin and SUMO samples, the ability for a minimum mass filter to enhance identification rates of ubiquitin and SUMO, and the ability of MQL to exclude either contaminant or all unmodified peptides in SUMO samples. Collectively, we find that defining a minimum precursor mass cutoff, compared to setting a minimum m/z, can increase MS selectivity of Ubl-modified peptides from low purity samples by up to 7-fold, and increase the number of identified ubiquitin and SUMO sites by up to 30% from the same samples, without the requirement for prior sample knowledge.

## METHODS

### Cell Lines

U-2 OS cells (HTB-96) were acquired via the American Type Culture Collection, and Hep2 cells and were a kind gift from Dr. Bo van Deurs, Copenhagen University. U-2 OS cells were cultured at 37 °C and 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM), GlutaMAX (Invitrogen) supplemented with 10% fetal bovine serum (FBS), and a penicillin/streptomycin mixture (100 U/mL; Gibco). Hep2 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 50 U/mL penicillin/streptomycin. Cells were routinely tested for mycoplasma. Cells were not routinely authenticated. For SUMO samples, one batch of U-2 OS cells was cultured under standard growth conditions, using 30×15 cm dishes, containing ~600 million cells and yielding ~150 mg of extracted total protein. Technical quadruplicate MS runs were performed on final purified material from ~3 mg of total protein. For ubiquitin samples, one batch of Hep2 cells was cultured under standard growth conditions, using 30×15 cm dishes, containing ~400 million cells and yielding ~100 mg of extracted total protein. Technical triplicate MS runs were performed on final purified material from ~0.5 mg of total protein (trypsin digest) or ~0.75 mg of total protein (Lys-C and LysC/Asp-N digest).

### Peptide Preparation for SUMO-IP

Sample preparation and subsequent immunoprecipitation of SUMOylated peptides was performed essentially as described previously.26 Briefly, U2OS cells were washed twice with ice-cold phosphate-buffered saline (PBS), after which they were scraped into ice-cold PBS and pelleted at 400g. Cells were vigorously lysed in 10 pellet volumes of 6 M guanidine-HCl, 50 mM TRIS, pH 8.5. The lysate was supplemented with 5 mM chloroacetamide (CAA) and 5 mM Tris(2-carboxyethyl)-phosphine (TCEP), and sonicated using one 10s and 30W pulse per 10 mL of lysate, with brief pauses in between. The sample was digested with Lys-C (Wako) at a 1:200 (w/w) enzyme-to-protein ratio, overnight at room temperature. The sample was diluted with 3 volumes of 50 mM ammonium bicarbonate (ABC), and afterward further digested with an additional 1:200 (w/w) of Lys-C. Digested lysates were
acidiﬁed by addition of trifluoroacetic acid (TFA), clariﬁed by centrifugation, and peptides were puriﬁed using C8 Sep-Pak cartridges (Waters) according to the manufacturer’s instruc-
tions. Nonrelevant small peptides were washed off with 25% acetonitriple (ACN) in 0.1% TFA, and large peptides were eluted using 4 mL 45% ACN in 0.1% TFA. Elutions were frozen at −80 °C and afterward lyophilized.

Puriﬁcation of SUMOylated Peptides
SUMO-IP beads were prepared as described previously,22 with 50 μL of SUMO-2/3 antibody (8A2, acquired from Abcam, ab81371; ~5–10 μg/μL antibody) conjugated to 100 μL of Protein G Agarose beads (Roche), using dimethyl pimelimidyl acid sodium bicarbonate pH 8.5 stock, and afterward digested with trypsin. The sample was divided into two equal parts. One part of the sample was adjusted to a ﬁnal concentration of 50 mM sodium bicarbonate, using a 1 M Na2HPO4, pH 7.2, centrifuge-cleared, after which 100 μL of SUMO-IP beads were added and the sample mixed at 4 °C for 3 h. Beads were washed 2× with ice-cold SUMO-IP buffer, 2× with ice-cold PBS, and 2× with ice-cold MQ water, after which the beads were eluted twice with 200 μL of ice-cold 0.15% TFA. Eluted SUMOylated peptides were pH-neutralized by addition of 1/10th volume of 1 M Na2HPO4, after which they were digested with 2 μg of Asp-N (Roche), overnight at 30 °C.

Puriﬁcation of Ubiquitylated Peptides
Preparation of peptides for ubiquitin-IP and puriﬁcation of ubiquitylated peptides from Hep2 cells was performed exactly as described previously.17 After elution of the immunoprecipi-
tated peptides with UbiSite beads the sample was divided into two equal parts. One part of the sample was adjusted to a ﬁnal concentration of 50 mM sodium bicarbonate, using a 1 M sodium bicarbonate pH 8.5 stock, and afterward digested with 2 μg of sequencing-grade trypsin (Promega), overnight at 37 °C.

StageTip Cleanup
C18 StageTips were prepared in-house.30,31 For all experiments, four plugs of C18 material (Sigma-Aldrich, Empore SPE Disks, C18, 47 mm) were layered per StageTip. Activation was performed with 100% methanol followed by 100 μL 80% ACN in 0.1% formic acid, equilibration twice with 100 μL 0.1% formic acid, after which samples were loaded. StageTips were washed twice with 150 μL 0.1% formic acid, subsequently eluted with 80 μL 30% ACN in 0.1% formic acid, and dried to completion in a SpeedVac at 60 °C. The SUMOylated peptide batch was dissolved in 150 μL 0.1% formic acid, of which 3 μL (2% total sample) was used per replicate injection for MaxQuantLive experiments, and 0.3 μL (0.2% total sample; diluted 10× with 0.1% formic acid) per replicate injection for Exploris experiments. Half of the trypsin-digested ubiquitin sample was puriﬁed by StageTip and afterward dissolved in 250 μL 0.1% formic acid, of which 5 μL (0.5% total sample) was used per replicate injection. Ubiquitin samples digested with Lys-C or Lys-C/Asp-N were puriﬁed and dissolved in 167 μL 0.1% formic acid, of which 5 μL (0.75% total sample) was used per replicate injection.

Mass Spectrometric Analysis
All samples were analyzed on 15 cm long 75 μm internal diameter columns, packed in-house with ReproSil-Pur 120 C18-AQ 1.9 μm beads (Dr. Maisch), connected to an EASY-nLC 1200 system (Thermo). MaxQuantLive (MQL) experi-
ments were performed using a Nanospray Flex Ion Source (Thermo) coupled to a Q Exactive HF-X Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo). SUMO Exploris experiments were performed using a Nanospray Flex NG ion source (Thermo) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo). A column heater was used to heat the column to 40 °C. Column elution was performed using Buffer A (0.1% FA) as the initial buffer, and an increasing amount of Buffer B (80% ACN in 0.1% FA) over time, depending on the sample type loaded. SUMO MQL experiments were analyzed using 80 min gradients, including ramp-up and washout, and an effective gradient ranging from 13 to 32% buffer B over 50 min. SUMO Exploris experiments were analyzed using 60 min gradients, with an effective gradient ranging from 13 to 32% buffer B over 36 min. Ubiquitin MQL experiments were analyzed using 80 min gradients, with effective buffer B ramps depending on the enzymes used. Trypsin: 8–34% over 56 min, Lys-C only: 16–34% over 50 min, Lys-C and Asp-N: 16–25% over 50 min. For all experiments, spray voltage was set to 2 kV, ion transfer tube temperature to 275 °C, and an RF funnel level to 40%. Full scans were made at a resolution of 60 000 (SUMO normal, ubiquitin normal and sensitive) or 120 000 (SUMO sensitive and ultra, ubiquitin ultra, Exploris). MS1 AGC target was set to 3 000 000 charges (”300” on Exploris), with a maximum injection time of 60 ms (for 60 000 resolution) or 120 ms (for 120 000 resolution), or “Auto” (Exploris). Scan ranges used (m/z) were as follows; SUMO: 400–1600, Lys-C or Asp-N ubiquitin: 300–1300, tryptic ubiquitin: 300–1200. Precursor fragmentation was achieved through higher-energy collision disassociation (HCD) with a normalized collision energy of 25, an AGC target of 200 000 (“200” on Exploris), and an isolation width of 1.3 m/z. Minimum MS2 AGC target was set to 20 000. Charges 2–5 were considered for MS/MS, with a dynamic exclusion of 80 s (for 80 min gradients) or 60 s (for 60 min gradients). Loop counts were set to 14 (ubiquitin normal), 7 (SUMO normal or ubiquitin sensitive), 5 (SUMO sensitive or ubiquitin ultra), 4 (SUMO sensitive Exploris), or 3 (SUMO ultra). MS2 resolution was set to 30 000 (ubiquitin normal) or 60 000 (all other experiments). Maximum MS2 injection times were as follows: 60 ms (ubiquitin normal), 120 ms (SUMO normal or ubiquitin sensitive), 360 ms (SUMO sensitive or ubiquitin ultra), 600 ms (SUMO ultra Exploris), or 1000 ms (SUMO ultra).

MaxQuantLive and Exploris Methodology Details
For the MQL experiments, the main method settings used were identical between Xcalibur and MaxQuantLive, with exceptions indicated below. In all cases, MaxQuant.Live control over the mass spectrometer was initiated and terminated by using “magic scans” as deﬁned in the MQL documentation.29 For all MQL runs, “LifeTime” was set to 5000 ms. On the basis of initial pilot experiments (data not shown) monitoring the number of charges observed in the Orbitrap (“RawOvFtT” value) when running with either Xcalibur or MaxQuant.Live instrument control, the MS2 AGC target for MQL runs was raised from 200 000 to 300 000 to prevent underinjection and achieve a comparable number of ions in the Orbitrap. For SUMO mass ﬁltering experiments, the “TopN” app28 in MQL was used to set a minimum ion mass of 1700 Da. For tryptic ubiquitin, 1000 Da. For Lys-C and Asp-N ubiquitin, 2200 Da. For SUMO exclusion experiments, the “Targeting” app28 in MQL was used, with default “RealtimeCorrection” settings. Contaminant peptides to be excluded were based on known human contaminants and mouse-only peptides detected in prior runs. To achieve this,
MS raw data were analyzed immediately and additional evidence was provided to subsequent replicate exclusion runs. The first replicate series exclusion list was based on the first two runs (Norm_Xcal_01 and Norm_MQL_01). The second replicate series exclusion list was based on the 10 runs from the first replicate. The third and fourth replicate series exclusion list was based on the 20 runs from the first and second replicate. All 40 runs were used to generate an exclusion list for the triplicate run where all non-SUMOylated peptides were excluded. The final contaminant (356 entries) and unmodified (7610 entries) exclusion lists are provided as Table S5. For tryptic ubiquitin experiments where a low m/z range was investigated, 200–1200 m/z was used instead of 300–1200 m/z. For MQL and Exploris experiments, intensity thresholds were defined for precursor selection, equivalent to and instead of minimum MS2 AGC target. The “Targeting” app in MQL did not include an intensity threshold setting. For the standard Exploris SUMO method, the precursor selection range was 100–1600 m/z, considering charges 2–5. For the “minimum mass” method, two alternating methods were used, the first targeting z = 3 with a precursor selection range of 566–1600 m/z, and the second targeting z = 4–5 with a precursor selection range of 425–1600 m/z. For the “minimum m/z” method, the quadrupole isolation range was set to 550–1600 m/z, and only charges 3–5 were considered.

**MS Raw Data Analysis**

All MS RAW data were analyzed using the freely available MaxQuant software, version 1.5.3.30. Data were analyzed in different computational runs depending on experiment type; SUMO MQL, ubiquitin tryptic, ubiquitin LysC, ubiquitin LysC/AspN, and SUMO Exploris. Default MaxQuant settings were used, with exceptions specified below. For generation of the theoretical spectral library, the human FASTA database was downloaded from Uniprot on the 23rd of July, 2018, and only used to add mouse exclusive contaminant peptides in the SUMO MQL data. For SUMO data, in silico digestion of theoretical peptides was performed with Lys-C, Asp-N, and Glu-N, allowing up to 8 missed cleavages (MC). For tryptic ubiquitin data: with trypsin up to 3 MC. For Lys-C ubiquitin data: with Lys-C up to 2 MC. For Lys-C/Asp-N ubiquitin data: with Lys-C, Asp-N, and Glu-N, up to 6 MC. Variable modifications (VMs) were used for protein N-terminal acetylation (default) and methionine oxidation (default). For SUMO searches, additional VMs were peptide N-terminal pyroglutamate, and Lys SUMOylation. The SUMO mass remnant was defined as described previously. DVFQQQTGG, H$_{160}$C$_{41}$N$_{15}$O$_{35}$ monoisotopic mass 960.4301, neutral loss b7-DVFQQQT, diagnostic mass remnants [b2-DV, b3-DVF, b4-DVFQ, b5-DVFQQ, b6-DVFQQQ, b7-DVFQQQT, b9-DVFQQQTGG, QQ, FQ, FQQ]. For tryptic ubiquitin data, additional VMs were Lys diglycine, and Lys LRGG. For Lys-C and Lys-C/Asp-N ubiquitin data, the long ubiquitin mass remnant was set as a VM and defined as described previously; ESTLHLVLRLRGG, H$_{109}$C$_{63}$N$_{21}$O$_{17}$, monoisotopic mass 1431.8310, and disallowed on peptide C-terminus. In all cases, a maximum of 3 modifications were allowed per peptide. Maximum peptide mass was set to 6000 Da, or 7000 Da for Lys-C only ubiquitin data. Stringent MaxQuant 1% FDR filtering was applied (default), and minimum delta score for modified peptides was increased to 20, with a site decay fraction of 2% (SUMO data) or 1% (ubiquitin data). Matching between runs was enabled, with a match time window of 1 min and an alignment window of 20 min. To further minimize false-positive discovery, additional manual filtering was performed at the peptide level. All modified peptides were required to have a localization probability of >75% and be absent in the decoy database. For SUMO data, modified peptides were also required to be supported by diagnostic mass remnants, and only allowed to reside on a peptide C-terminal lysine if preceding an aspartic acid or glutamic acid. Multiply SUMOylated or ubiquitylated peptides were discarded.

**RESULTS**

**Analysis of Endogenous SUMO Samples**

We previously established the first proteomics methodology for purification of peptides modified by native and endogenous SUMO2/3 (Figure 1B). While successful in identifying thousands of lysines modified by SUMO2/3, there are several inherent challenges related to peptide purity (Figure 1A) and the cumbersome nature of the 1 kDa SUMO2/3 C-terminal remnant (Figure 1B). In order to compare Xcalibur to MQL, we first utilized our SUMO-IP method to purify a batch of SUMO-modified peptides from untreated U2OS cells growing under standard cell culture conditions (Figure 1A), which served as a technical standard for further experiments. To ascertain a competitive comparison, the generated SUMO batch was split into aliquots and for each replicate we analyzed a very low amount of sample, corresponding to ~10 ng of SUMOylated peptides purified from ~10 million cells. We performed technical quadruplicate measurements, in which we compared Xcalibur and MQL head-to-head, using a standard and a sensitive measurement strategy (Figure 1C and Figure S1A).

Specifically, for both Xcalibur and MQL the standard measurement strategy employed here closely mimics previously used acquisition strategies, aimed at maximizing accuracy of PTM localization via recording MS1 and MS2 at 60 000 resolution and allowing up to 120 ms MS2 precursor injection time, resulting in a ~1 s duty cycle with ~6.3 high-resolution MS2 scans per second. The sensitive strategy utilized an MS1 resolution of 120 000, and allowed up to 360 ms MS2 precursor injection time, resulting in much higher sensitivity but with a duty cycle of up to ~2 s and only ~2.3–5.1 MS2 scans per second. In all cases, we considered charges 2–5, as z = 2 SUMO-modified peptides exist. Notably, SUMO-modified branched peptides are often observed with charge 3 and exclusion of charge 2 has been performed in the context of ubiquitin and SUMO proteomics.

For MQL, we additionally investigated a minimum mass setting of 1700 Da, representing the SUMO2/3 mass remnant size in addition to the minimum identifiable target peptide size of ~700 Da. We additionally considered exclusion of global contaminant peptides, which we defined as known human contaminant or mouse-unique peptides originating from the mouse antibody used for the IP, and which were detected during the prior runs. Finally, after all 40 runs were completed, we performed another triplicate measurement where we used MQ to exclude every non-SUMOylated peptide detected up to that point (Figure 1C), in this case using an ultrasensitive measurement strategy (Figure S1A).
The average mass of all identified peptides remained unaffected between Xcalibur and MQL analyses, and by the sensitivity of the measurement strategy (Figure 1D). However, defining a minimum mass dramatically increased the measured mass of peptides, indicating that this feature works remarkably well. In terms of spectral quality of all identified SUMOylated peptides (Figure 1E), we did not find any significant differences between usage of Xcalibur or MQL, but the more sensitive measurement strategies resulted in a large boost in spectral quality. An average of 961 SUMO peptide-spectrum matches (PSMs) and 658 unique SUMO sites were detected across all experiments (Table S1), representing a competitive number of SUMO sites identified from limited
starting material and without relying on offline prefractionation. When investigating the number of SUMO-modified PSMs (Figure 1F), we observed no notable differences between Xcalibur and MQL when using the standard measurement sensitivity, although at baseline settings there was a trend for MQL to outperform Xcalibur. Intriguingly, when using the more sensitive measurement strategy in combination with MQL precursor mass filtering, the number of SUMO PSMs increased by ∼28%, highlighting the analytical advantage of setting a minimum precursor mass (Figure 1F). The increase at the SUMO site level was comparable while using precursor mass filtering, with ∼23% more sites being identified (Figure 1G).

Investigation of various peptide properties revealed that the precursor mass filtering strategy effectively mitigated sequencing of nonmodified peptides while boosting detection of SUMOylated peptides (Figure S1B−E). Notably, when additionally considering MS1-level matching between runs (MBR) in the data search, we observed no significant differences in the number of SUMO sites identified between the different runs, indicative of an equal sample load (Figure 1H).

Figure 3. Evaluation of minimum precursor mass filtering for analysis of ubiquitylated peptides from Lys-C digest. (A) Experimental design overview. Minimum mass was set to 2200 Da. n = 3 technical replicates. Upper left: Carboxy-terminal sequence of ubiquitin. Arrows indicate where the specified proteolytic enzymes cleave the ubiquitin sequence. (B) Boxplot overview of the mass distribution of all identified peptides from Lys-C digest. Black line, median; black dashed line, average; box limits, 1st and 3rd quartile; whiskers, 5th and 95th percentile. Number of data points are indicated next to the boxplots. Labels: “Xcal”, Xcalibur; “M”, minimum mass filtering; “N”, normal; “S”, sensitive; “U”, ultrasensitive. (C) As (B), but for peptides from Lys-C/Asp-N digest. (D) Overview of the average relative number of ubiquitin-modified PSMs identified, from Lys-C or Lys-C/Asp-N digest. Labels: First row, “N”, normal; “S”, sensitive; “U”, ultrasensitive. Second row, “M”, minimum mass filtering. Third row, “M”, MQL; “X”, Xcalibur. Error bars represent SD, *p < 0.05, **p < 0.001, via two-tailed Student’s t-testing. (E) As (D), but showing the average number of ubiquitin sites identified via MS/MS. (F) As (D), but showing the average number of ubiquitin sites with matching between runs enabled. (G) As (D), but showing the average measured ubiquitin-modified peptide purity at the PSM-level.
more SUMOylated peptides, potentially due to a lower sample load (Figure 1H), but we did observe a striking ~90% SUMO-modified peptide purity (Figure 1I), demonstrating that MQL is extremely efficient at excluding known precursors. Overall, MQL in combination with precursor mass filtering and an appropriately sensitive measurement strategy was able to increase the SUMO identification rate from low-yield samples by nearly 30%, without the requirement for prior knowledge of the sample.

Analysis of Trypsin-Digested Ubiquitin Samples

With MQL able to boost the number of SUMO sites identified by setting a minimum precursor mass, we wondered whether the same strategy could be applied to ubiquitin. To investigate this, we utilized a recently described methodology for purification of ubiquitylated peptides after an initial Lys-C digest of the sample (Figure 2A),17 and purified ubiquitin from untreated Hep2 cells. Similar to the SUMO purification method, the final peptide mixture may be subjected to a second proteolytic enzyme after the ubiquitin-IP. This is usually trypsin but alternatively Asp-N, resulting in a different composition and purity of the final peptide mixture, and a difference in size of the ubiquitin mass remnant.

We initially considered the commonly utilized tryptic strategy (Figure 2B), yielding the small diglycine (GG) remnant,18 or alternatively the Leu-Arg-Gly-Gly (LRGG) remnant via one missed tryptic cleavage. Similar to the SUMO experiments (Figure 1), we prepared a batch of ubiquitylated peptides to serve as a technical standard, and performed technical triplicate measurements comparing a normal and sensitive measurement strategy (Figure S2A). Specifically, for both Xcalibur and MQL the standard measurement strategy recorded MS1 at 60 000 and MS2 at 30 000 resolution, and allowed up to 60 ms MS2 precursor injection time, resulting in a ~1 s duty cycle with ~12.1 MS2 scans per second. The sensitive strategy allowed up to 120 ms MS2 precursor injection time, resulting in much higher sensitivity but only ~6.3 MS2 scans per second. A relatively low amount of sample was analyzed, with each replicate corresponding to ~50 ng of ubiquitylated peptides purified from ~2 million cells. We evaluated setting a minimum mass of 1000 Da via MQL, striking a middle ground between GG and LRGG mass remnant size in addition to the minimum target peptide mass. Additionally, as ubiquitylated peptides can be relatively small while also highly charged, we investigated isolation of precursors from a lower m/z range (Figure 2B).

We first investigated the average mass of all identified peptides (Figure 2C), and observed no significant difference between any of the investigated strategies, suggesting that the additional mass resulting from ubiquitin does not notably change the size or m/z distribution of the analyzed peptides. In terms of average spectral quality of ubiquitylated peptides, there were no significant differences between Xcalibur and MQL, and only the sensitive measurement strategy significantly boosted spectral quality (Figure 2D). Across all single-shot analyses, we identified on average 1617 GG PSMS and 1310 GG sites per run, on average 1067 LRGG PSMS and 731 LRGG sites per run, and in total 3849 unique tryptic ubiquitin sites (Table S2). The average number of identified ubiquitin-modified PSMS and ubiquitin sites were overall lower in MQL compared to Xcalibur (Figure 2E,F), contrary to the trend observed for SUMOylated peptides (Figure 1), suggesting deviating baseline performance between MQL and Xcalibur. When using the sensitive measurement strategy, MQL with precursor mass filtering did significantly increase the number of ubiquitin PSMS and sites compared to baseline MQL (Figure 2E,F), especially for peptides modified with the LRGG remnant. However, despite an equal sample load (Figure 2G), the performance of the slower sensitive method was considerably worse compared to the standard method. We did not note any differences from setting the lower end of the precursor isolation range to 200 m/z. Inspection of technical properties of peptide populations highlighted no striking differences in the context of precursor mass filtering (Figure S2B–E), although a higher selectivity was observed at the PSM level for both GG and LRGG remnants. Ubiquitin-modified peptide purity was boosted by ~10% in all cases when using MQL precursor mass filtering (Figure 2H), indicating an overall enhancement in precursor selectivity, but ultimately with minor practical impact.

Analysis of Lys-C-Digested Ubiquitin Samples

Although the GG remnant may be too small to yield practical benefit when setting a minimum precursor mass filter, the ubiquitylated peptides as purified by our strategy may also be measured directly after Lys-C digest and purification, where they retain a ~1.4 kDa mass remnant (Figure 3A).17 To investigate this, we performed another technical triplicate set of experiments, where we utilized MQL to set a minimum mass cutoff of 2200 Da, and analyzed peptides using a standard, sensitive, or ultrasensitive strategy (Figure S3A). The standard and sensitive measurement strategies were identical with those used for the tryptic ubiquitin samples, with the ultrasensitive strategy recording MS1 at 120 000 and MS2 at 60 000 resolution, allowing up to 360 ms MS2 precursor injection time, resulting in a ~1–2 s duty cycle with ~2.3–5.1 MS2 scans per second. Furthermore, we included a sample of ubiquitylated peptides additionally digested with Asp-N, which reduces the overall length of the target peptides but does not affect the ~1.4 kDa ubiquitin mass remnant.

Using MQL precursor mass filtering on the large-remnant ubiquitin peptides demonstrated a remarkable shift in average peptide mass (Figure 3B), even though the Lys-C digest also yielded comparatively large unmodified peptides. Additional digestion with Asp-N strongly reduced the average mass of all precursors (Figure 3C), albeit to a somewhat lesser degree when MQL precursor mass filtering was enabled. Spectral quality was not notably different between Xcalibur and MQL, and as before only the increasingly sensitive measurement strategies had a large impact on average spectral score (Figure S3B,C). On average from the Lys-C digest, 937 ubiquitin PSMS and 635 ubiquitin sites were identified, with 1482 ubiquitin sites identified in total (Table S2). From the Lys-C/Asp-N digest, on average 413 and 298 ubiquitin PSMS and sites were mapped, respectively, with 744 unique ubiquitin sites identified overall (Table S2). In terms of ubiquitin-modified PSMS and ubiquitin sites (Figure 3D,E, left panels), MQL with precursor mass filtering provided a small and significant increase in performance for the higher sensitivity methods. In case of the Lys-C and Asp-N double-digested samples, which inherently were of lower purity owing to the introduction of more unmodified small peptides, MQL with precursor mass filtering identified considerably more ubiquitin sites, especially at the higher sensitivity levels (Figure 3D,E, right panels). With MBR showing equal sample load all across (Figure 3F), we finally investigated perceived ubiquitin-
modified peptide purity. Here, MQL precursor mass filtering resulted in a ~50% relative increase in peptide purity in case of the Lys-C samples, and a striking 4- to 7-fold relative increase for Lys-C/Asp-N samples, corresponding to an increase from ~4% to ~27% absolute peptide purity (Figure 3G). For both Lys-C and Lys-C/Asp-N samples, precursor mass filtering efficiently reduced sampling of unmodified peptides (Figure S4). Taken together, we profiled a total of 4554 unique ubiquitin sites across all proteolytic digestions and ubiquitin mass remnants (Figure S3D and Table S3), with 2688 (59%) sites exclusively detected in one condition, and 210 (4.6%) sites detected in all four conditions. Our data suggest that MQL with precursor mass filtering might only provide a minor advantage to analysis of standard diglycine ubiquitin samples, but could yield a considerable benefit for analysis of Lys-C-digested ubiquitin peptides from lower purity samples.

Properties of SUMO- and Ubiquitin-Modified Peptides

With all the data acquired on SUMO- and ubiquitin-modified peptides (Figures 1–3), we set out to investigate the properties of these peptides compared to the unmodified peptides within the same samples, to enable further optimization of instrument methodology. We first investigated the distribution of m/z for all unmodified and modified peptides found in all experiments (Figure 4A), with m/z being one of the primary characteristics when configuring contemporary MS approaches. Interestingly, we observed that the m/z of small-remnant ubiquitin peptides was somewhat lower than the m/z of unmodified peptides in the same sample, likely because of the additional N-terminus carrying charge in acidic buffers, while the added mass is minimal. For the large-remnant ubiquitin peptides, no difference was observed versus unmodified peptides from the same sample, although additional digestion with Asp-N notably lowered m/z of the unmodified peptides. For SUMO-modified peptides, the observed m/z was considerably higher compared to unmodified peptides, suggesting that a higher m/z cutoff for precursor selection could also be beneficial. When considering the average mass of all peptides observed in our experiments (Figure 4B), we observed the logical trend for modified peptides to be larger than unmodified peptides from the same sample, but the most dramatic size differences were observed for both SUMO and ubiquitin in case of Lys-C/Asp-N digestion. Notably, the mass for unmodified peptides from the ubiquitin Lys-C-only digest was not much lower compared to the ubiquitylated peptides, because the observed unmodified peptides were much longer (Figure S5A).

Figure 4. Analysis of SUMO- or ubiquitin-modified peptide properties. (A) Boxplot overview of the m/z distribution of all identified unique peptides from all experiments. Black line, median; black dashed line, average; box limits, 1st and 3rd quartile; whiskers, 5th and 95th percentile. Number of data points are indicated next to the boxplots. Labels: First column, “LC”; Lys-C; “AN”, Asp-N; “TR”, trypsin. Second column, “9aa”, 9 amino acid SUMO remnant; “13aa”, 13 amino acid ubiquitin remnant. Third column, “+” indicates modified peptides. (B) As (A), but visualizing mass distribution. (C) Overview of m/z and mass ratios between modified and unmodified peptides. n = 4 ratio calculations based on the entire peptide populations’ average, 40th, 50th, and 60th percentile m/z and mass values. Error bars represent SD. Labels as (A). (D) Global performance analysis of minimum mass filtering, based on data presented in Figures 1–3. Labels: First row, “M”, minimum mass filtering. Second row, “M”, MQL; “X”, Xcalibur. n = 8 for SUMO, n = 12 for trypsic digest ubiquitin, n = 9 for Lys-C digest ubiquitin, with values pairwise normalized within similar experimental conditions. Error bars represent SEM, *p < 0.05, **p < 0.001, via two-tailed Student’s t-testing.
for using contemporary method design approaches, e.g., by increasing the minimum $m/z$ for precursor selection. To this end, we pairwise normalized the experimental values obtained for all PTM sites thus far for each measurement sensitivity, and created one overarching performance readout (Figure 4D). Indeed, we observed overall performance boosts via MQL precursor mass filtering which correlated quite well to the difference between mass and $m/z$ ratios (Figure S5B), with
significant performance increases of ~20% for SUMO, ~10% for ubiquitin, and ~30% for ubiquitin from Lys-C/Asp-N double digest.

**Analysis of SUMO on Exploris 480**

During preparation of this manuscript, the Orbitrap Exploris 480 mass spectrometer was released, while MaxQuant.Live only supported the Q-Exactive line of mass spectrometers. The Exploris, however, features an updated method editor allowing for the construction of more elaborate MS methods, although the minimum precursor mass filtering as performed by MQL was unavailable. Nonetheless, we were keen to apply some of the lessons learned from MQL to the analysis of endogenous SUMO-modified peptides on the Exploris.

We designed an experiment comparable to the SUMO experiment performed on the Q-Exactive HF-X (Figure 1A–C). As the Exploris exhibits improved sensitivity, we further lowered the amount of input material by 10-fold to ~1 ng of purified SUMOylated peptides, in order to approach the detection limit of the Exploris. Notably, the amount loaded was comparable to the total protein amount in a single HeLa cell. We measured technical quadruplicates at three levels of sensitivity (Figures 5A and S6A), with the general instrument settings highly comparable to what we used for SUMO analysis (Figures 5A and S6). We were able to utilize the ability of MQL to target, or exclude precursors in an unbiased manner, and in total identify 725 endogenous SUMO sites (Figure 5D,E), with equal loading levels confirmed via MBR (Figure SF). However, using the sensitive alternative approaches we observed an increase of ~45% or ~21% in the number of SUMO sites identified compared to the baseline standard or sensitive methods, respectively. The minimum m/z range method remained competitive even when operating the instrument at the comparatively slow ultrasensitive level, where the baseline method performed worse. We also noted a striking and consistent increase in perceived SUMO-modified peptide purity across all levels of sensitivity (Figure 5G), with the PSM-level purity increasing from ~28% baseline to ~57% with the alternative Exploris methods, overall performing very similar to the MQL precursor mass filtering method.

**DISCUSSION**

In this study, we investigated the ability of precursor mass filtering as uniquely enabled by the MaxQuant.Live software to optimize the real-time MS acquisition of SUMOylated and ubiquitylated peptides. Our goal was to enhance selectivity toward modified precursors in an unbiased manner, and without the requirement for prior knowledge such as spectral libraries which are typically required for data-independent acquisition (DIA) approaches, although progress is being made toward establishing library-free DIA. Primarily, the ability of MQL to set a minimum precursor mass filter allowed the MS to ignore precursors with low masses, regardless of their charge states. Second, we considered the targeting ability of MQL to exclude global contaminant peptides. Third, we applied some of the lessons learned to the new Orbitrap Exploris 480, in order to maximize selectivity of SUMOylated precursor selection.

During our investigation of SUMO-modified peptides, we found a definitive benefit of using precursor mass filtering by MQL on the Q-Exactive HF-X (Figure 1). The increased number of SUMO sites identified was only observed when using a more sensitive measurement strategy as compared to the standard measurement approach. We reason that this is primarily because the faster method is limited in precursor isolation time, which combined with only targeting large peptides, resulted in the instrument running out of precursors to isolate. The same faster method without precursor mass filtering was able to isolate and identify smaller peptides without missing a significant number of modified peptides. As anticipated, when switching to a more sensitive measurement strategy, the additional precursor isolation time allowed the instrument to reach deeper into the large-peptide range, boosting the number of identified SUMO sites. Using the Exploris, we observed a similar trend, with a high degree of instrument sensitivity required to benefit from only targeting large peptides (Figure 5).

We were able to utilize the ability of MQL to target, or rather exclude, specific peptides. In our case, we first tried to exclude common human contaminant peptides or those derived from the mouse antibody used to IP the initial SUMO peptides. Overall, we did not observe any improvement in identification rate, and even found a trend for the exclusion methods to perform worse (Figure 1). At the time of performing our experiments, the “Targeting” app in MQL, which is required to specifically target or exclude precursors, did not have a minimum ion threshold setting, unlike the “TopN” app we used for the other MQL runs. Essentially, this meant that the instrument would always initiate scans even when the number of available precursor ions were insufficient for a tandem-MS scan to successfully identify the peptide, ultimately resulting in many doomed scans being initiated, with these precursors subsequently excluded from being re-sequenced. Second, we note that the overall list of contaminant...
peptides to exclude was relatively short (356 entries) compared to the overall number of peptides identified. However, when we investigated the ability for MQL to exclude all (7610) previously identified unmodified peptides, we found that MQL is exceptionally precise and was able to increase the perceived SUMO-peptide purity to >90% from a sample that in practice was only ∼25−27% pure at the PSM-level (Figures S1 and S1F). Thus, with knowledge of unmodified and contaminant peptides, MQL would be able to dedicate all precursor injection time to unknown peptides-of-interest, while otherwise still operating in data-dependent acquisition (DDA) mode.

Ubiquitin-modified peptides proved more challenging to specifically target with a minimum precursor mass setting (Figures 2–3). The diglycine remnant, one of the most routine ways to investigate ubiquitin-modified peptides, or alternatively SUMO-modified peptides, only adds 114.04 Da to the peptide size. However, in the context of positive-mode mass spectrometry, an extra charge is introduced via the second N-terminus resulting from ubiquitin, overall producing a higher mass (Figure 4). The tryptic digestion does not always release the diglycine, and we observed a notable occurrence of the LRGG mass remnant in our samples (Figure 2), where ubiquitin was purified after an initial Lys-C digestion. Between both these mass remnants, we found that the LRGG remnant was most compatible with a minimum mass filter, likely because of the additional mass and the introduction of two positive charges via the internal arginine and N-terminus. With both small ubiquitin remnants lowering m/z even though they have a higher mass (Figure 4). The tryptic digestion does not always release the diglycine, and we observed a notable occurrence of the LRGG mass remnant in our samples (Figure 2), where ubiquitin was purified after an initial Lys-C digestion. Between both these mass remnants, we found that the LRGG remnant was most compatible with a minimum mass filter, likely because of the additional mass and the introduction of two positive charges via the internal arginine and N-terminus. With both small ubiquitin remnants lowering m/z while raising mass, there is a potential small benefit to be gained from integration of a minimum mass setting into contemporary methods.

Investigation of the large 1.4 kDa ubiquitin remnant resulting from Lys-C digestion yielded only a modest benefit (Figure 3). Here, we propose that this is because the Lys-C digest also generated very large unmodified peptides (Figure 4B), making it much harder to separate the ubiquitin-modified peptides from the unmodified peptides when filtering by mass. While we expected the modified peptides to be even larger, we did not observe this propensity in practice, and contrarily found that the unmodified peptides were of similar size because they were much longer (Figure S5). Ubiquitin is likely to inhibit cleavage of lysine residues by Lys-C, with derivatization of lysine residues in target proteins abolishing Lys-C activity, so we would expect modified peptides to be considerably larger. Thus, we reason that many of the ubiquitin-modified peptides may be larger than 4 kDa where MS/MS identification rates sharply decline, thereby artificially lowering the average mass of the ubiquitin-modified peptides we detected in this case. Indeed, secondary digestion of the samples with Asp-N, which does not affect the 1.4 kDa ubiquitin remnant, largely normalized our observations, and introduced a large size difference between modified and unmodified peptides (Figures 4B and S5).

Additionally, we investigated the ability of the Exploris mass spectrometer to mimic the minimum precursor mass filtering option of MQL, although the Exploris does not natively support such an approach. Instead, we utilized an isolation range targeted at much higher m/z, and specifically isolated only charges 3−5. Indeed, this excluded nearly 10% of SUMOylated peptides from being detectable, as these can also be doubly charged or have an m/z lower than where we defined the cutoff. However, our data (Figures S1B–E, S6B−E), as well as previous reports, indicate that z = 2 is relatively underrepresented for SUMOylated peptides, and thus exclusion of z = 2 could be considered a viable strategy for promoting isolation of SUMOylated precursors. We aimed at maximizing the fraction of SUMOylated peptides within the m/z isolation range on the Exploris, and were able to successfully reach a SUMO-modified peptide purity on par with MQL precursor mass filtering. While some precursors may be impossible to detect with such a methodology, the overall increase in the number of identified SUMO sites is likely to compensate.

The success of minimum precursor mass filtering via MQL appears correlated to the difference in the mass and m/z ratios between the unmodified and modified peptides (Figure 4C,D and SSB). Ubiquitin and SUMO, and perhaps other ubiquitin-like modifications, are ideal targets of this approach since their mass remnants carry additional charge, often dampening the m/z gain compared to the mass gain. Other large and bulky PTMs, such as ADP-ribosylation, might potentially benefit from our approach under the assumption that these PTMs interfere with nearby proteolytic cleavage of basic residues, which would increase peptide positive charge and could inflate mass more than m/z. We investigated SUMO and ubiquitin samples purified from cancer cell lines, resulting in a fairly high peptide purity of ∼25%, and still managed to see a consistent benefit from mass filtering. It is likely that the minimum mass filtering approach described here would be increasingly beneficial on less pure samples, e.g., when the PTMs are purified from limited material or biologically challenging samples, essentially as demonstrated by the Lys-C/Asp-N double digest which was only ∼4% pure (Figure 3). Conversely, samples with a very high modified-peptide purity (e.g., >75%) would not benefit from this approach as most scans would already target peptides of interest.

In summary, native minimum precursor mass filtering by MaxQuant.Live increased the number of identified ubiquitin and SUMO sites by ∼10−30% and ∼20%, respectively, and we were able to emulate a similar increase in SUMO site identification using the Exploris. MaxQuant.Live was able to selectively exclude unmodified and contaminant peptides and elevate the isolation selectivity of SUMO-modified peptides to ∼90% from a ∼25% pure sample. Taken together, our minimum mass filtering strategy is able to significantly increase identification of SUMOylated and ubiquitylated peptides in an unbiased manner and while operating in a data-dependent acquisition mode, without the need to adjust sample preparation methods, and may similarly be able to boost identification rates of other bulky and obscure post-translational modifications.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00892.

Table S1: List of all SUMO sites (XLSX)
Table S2: List of all ubiquitin sites per experiment (XLSX)
Table S3: List of all unique ubiquitin sites (XLSX)
Table S4: SUMO sites identified with an Exploris 480 (XLSX)
Table S5: MaxQuant.Live exclusion lists (XLSX)

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Figure S1: SUMO MaxQuant.Live experimental and peptide details; Figure S2: Ubiquitin (trypsic) MaxQuant.Live experimental and peptide details; Figure S3: Ubiquitin (Lys-C) MaxQuant.Live experimental details; Figure S4: Ubiquitin (Lys-C) MaxQuant.Live peptide details; Figure S5: Analysis of SUMO- or ubiquitin-modified peptide properties; Figure S6: SUMO Explorls 480 experimental and peptide details (PDF)

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**Author Contributions**

I.A.H. and M.L.N. conceived the minimum mass filtering strategy. I.A.H. prepared SUMO samples. V.A. prepared ubiquitin samples. I.A.H. processed samples for MS measurement, performed MS experiments, analyzed MS data, and prepared the first draft of the manuscript. M.L.N and B.B. supervised the project. I.A.H. and M.L.N. finalized the manuscript with input from all authors.

**Notes**

The authors declare no competing financial interest. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE 

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