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REVIEW

1,2,3, MHC: a review of mass-spectrometry-based immunopeptidomics methods for relative and absolute quantification of pMHCs

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Quantitative mass-spectrometry-based methods to perform relative and absolute quantification of peptides in the immunopeptidome are growing in popularity as researchers aim to measure the dynamic nature of the peptide major histocompatibility complex repertoire and make copies-per-cell estimations of target antigens of interest. Multiple methods to carry out these experiments have been reported, each with unique advantages and limitations. This article describes existing methods and recent applications, offering guidance for improving quantitative accuracy and selecting an appropriate experimental set-up to maximize data quality and quantity.

Key words: HLA, MHC, immunopeptidomics, mass spectometry, antigen presentation

INTRODUCTION

Immune cell recognition of peptides presented by class I and class II major histocompatibility complex (MHC) plays a critical role in regulating the immune response. The collection of peptide MHCs (pMHCs) presented on the cell surface is commonly referred to as the 'pMHC repertoire', 'ligandome' or 'immunopeptidome'; correspondingly, 'immunopeptidomics' has emerged as a term to describe analysis of the pMHC repertoire. The most common immunopeptidomics methods rely on mass spectrometry (MS) as it is able to identify thousands of unique pMHCs in a single analysis in a relatively unbiased manner.[1] Immunopeptidomics samples are generally prepared by isolating MHCs using an allele-specific antibody,[2] pan-specific antibody[3] or engineered affinity tag system[4,5] from lysed cells or tissues (Figure 1A). Isolated complexes are acid eluted, and peptides are purified from the MHC using molecular weight cut-off filtration (MWCO), solid phase extraction or other techniques, and are subsequently analysed by MS.

To date, MS-based datasets have identified hundreds of thousands of unique pMHCs across a variety of cell, tissue, species and disease types,[6-8] yet quantitative information regarding pMHC expression is typically missing from these data. There is growing interest in applying quantitative MS methodologies commonplace in standard bottom-up proteomics workflows to immunopeptidomics, as the pMHC repertoire is an external representation of the internal cell state, and changes in cell state are reflected in quantitative differences in the immunopeptidome. Datasets evaluating repertoire changes across timescales, tissues and perturbations will enhance our understanding of mechanisms regulating which peptides are processed and presented, and may reveal new or differentially presented epitopes that can be utilized as therapeutic targets.

To access this information, methods enabling relative quantification of pMHC abundances between two or more samples have emerged; more recently, these methods have been extended to enable estimates of the absolute abundance of pMHCs. Achieving absolute quantification of presented peptide antigens, a copies-per-cell estimate, is necessary to inform immunotherapy drug design, as targeted strategies depend on varying thresholds of target cell antigen presentation for an optimal antitumor response.[9-11] Multiple MS-based methodological approaches for relative and absolute quantification have been reported, each with unique advantages and limitations relating to quantitative accuracy, data quantity, throughput, cost, ease of use, and more, that should be considered prior to selecting an experimental approach. This article describes an overview of existing methods and recent applications, and suggest best practices for generating accurate relative and absolute quantitative immunopeptidomics datasets.

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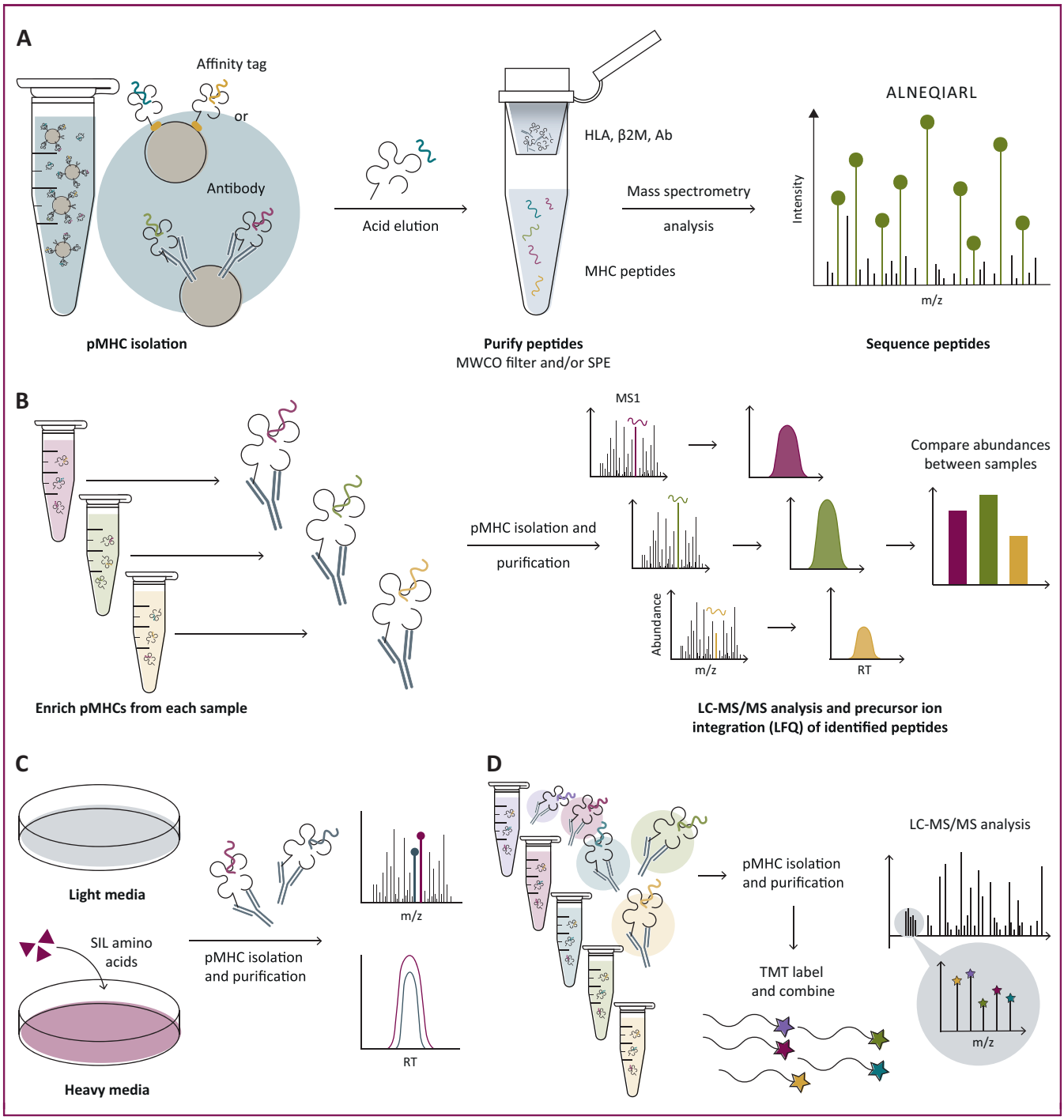

Figure 1. Schematics of immunopeptidomics workflows for standard discovery-based peptide major histocompatibility complex (pMHC) identifications (A), label-free quantification (LFQ) (B), stable isotope labeling (SIL) using amino acids (C), and multiplexed quantification with isobaric mass tags (D).
MWCO, molecular weight cut-off; SPE, solid phase extraction; MS, mass spectrometry; TMT, tandem mass tag; RT, retention time; LC-MS/MS, liquid chromatography with tandem mass spectrometry.

RELATIVE QUANTIFICATION OF PMHCS BY MASS SPECTROMETRY

Label-free relative quantification

Label-free quantification (LFQ) is the most common pMHC quantitative method. In LFQ, the samples being compared are analysed individually; quantitation is typically performed by integrating the area under the chromatographic elution of the precursor ion in discovery mode analyses, although product ions may be used in the case of targeted analyses (Figure 1B). Over the past decade, many authors have utilized LFQ to measure differences in the immunopeptidome. In 2011, Caron et al. published a quantitative dataset which displayed altered pMHC abundances in response to rapamycin therapy, and described systems-level evidence for the immunopeptidome serving as an external

representation of the cell state.[12] Several studies have probed how interferon gamma (IFN-γ) stimulation alters the pMHC repertoire across human and mouse studies, and similarly found that IFN-γ-related pMHCs are upregulated in presentation following stimulation.[13-17] Other reports using LFQ have explored how perturbations such as small molecule inhibitors[15] and oncolytic retroviruses[18] shape the immunopeptidome *in vitro* and *in vivo*, and how different single-cell-derived tumor organoids reveal interclonal variability.[19] Together, these data reveal therapeutically targetable pMHCs, and serve to better inform our understanding of the dynamic pMHC repertoire across different samples and conditions.

While LFQ offers the opportunity to compare quantitation across a theoretically unlimited number of samples and is straightforward to implement, it has several notable limitations. First, data-dependent acquisition methods suffer from poor overlap in peptide identifications across analyses. Previously, using LFQ across six biological replicate samples, it was shown that <44% of peptides were quantifiable across all analyses.[20] While the overlap across analyses may be improved slightly by calculating abundance values for precursor ions in the absence of an associated MS^2 spectrum, this can result in false-positive data points, particularly because pMHCs from a given allele tend to have similar mass-to-charge ratios and biological properties.[15,21,22] Relative quantitation among class II pMHCs adds an additional layer of complexity, as class II peptides typically form nested sets of varying lengths, and require peptides to be grouped into consensus epitopes to compare quantitation.[23,24] As an important consideration, variations in sample input and processing between samples can result in variable and inaccurate quantitation in LFQ. Previously, it was reported that recombinant MHC monomers loaded with heavy isotopically-labeled MHC peptides (hipMHCs) using ultraviolet-mediated peptide exchange[25] can be added to samples prior to immunoprecipitation (IP), and used as an internal standard to correct for variation between samples to circumvent this limitation.[20] Application of hipMHC correction factors improved quantitative accuracy, and reduced the median coefficients of variation between replicate analyses.

Multiplexed relative quantification

To minimize run-to-run variability that can have an adverse effect on label-free analyses, labeling strategies such as stable isotope labeling using amino acids in cell culture (SILAC),[26] in which cells from one condition are cultured in the presence of heavy-isotope-labeled amino-acid-containing media and combined with cells from another condition cultured in light-isotope-labeled amino-acid-containing media, have been used (Figure 1C). SILAC quantification is performed by comparing the light-to-heavy ratio of precursor ion intensities within a single analysis. As samples are mixed prior to enrichment of pMHC, SILAC minimizes the variability associated with processing different samples. However, the multiplexing capacity is limited to just a few samples, and application of SILAC to in-vivo samples can be challenging and fairly expensive.[27,28] Furthermore, SILAC is not optimized for most immunopeptidomics experiments, as MHC alleles are not restricted to a single amino acid at a given site, so standard SILAC heavy lysine/arginine-containing reagents will label pMHCs incompletely. However, SILAC media has been used in pulse-chase experiments to profile protein turnover kinetics of MHC molecules, and to study cross-presentation of tumor peptides on dendritic cells *in vitro*.[29-31] In these studies, leucine has typically been selected as the heavy-labeled amino acid, as it is an anchor residue in class I HLA-A*02:01 peptides. Custom SILAC media containing multiple heavy-labeled amino acids can be used to achieve higher labeling coverage of anchor residues, although it is challenging to achieve complete labeling given the diversity of peptide sequences that bind to any given allele.[4,31]

An alternative approach to SILAC labeling is to utilize isobaric mass tags, allowing for the multiplexed quantitation of up to 18 samples within a single analysis.[32] In this strategy, peptides are labeled with isobaric mass tags after isolation and combined prior to analysis, minimizing missing values (Figure 1D). Early studies by Bogunovic et al. and Shetty et al. utilized isobaric tags for relative and absolute quantification to multiplex up to four samples, demonstrating the utility of measuring repertoire alterations for different conditions.[33,34] Murphy et al. first extended this approach to tandem mass tags (TMT), multiplexing 10 samples to measure the immunopeptidome's response to doxorubicin treatment *in vitro* and *in vivo*.[35] More recent applications of this approach include using TMT to investigate the dynamics of HLA presentation following viral infection with severe acute respiratory syndrome coronavirus-2, and to quantify how perturbations in expression and degradation alter neoantigen expression.[36,37]

While these applications demonstrate the potential for using isobaric mass tags for pMHC quantitation, variations in sample amounts or sample processing can have an adverse effect on quantification, especially as samples are labeled and mixed after pMHC enrichment. To address this issue, hipMHCs were added to samples prior to pMHC enrichment as a normalization strategy across multiplexed analyses, and improved quantitative accuracy with hipMHC correction was demonstrated.[20] This method was used to profile pMHC repertoire responses to CDK4/6 inhibition and IFN-γ stimulation, which highlighted treatment-induced changes in pMHC presentation that may be leveraged with combination therapy strategies similar to Murphy et al.[20]

Multiplexed relative quantitation with isobaric mass tags such as TMT has several unique advantages over LFQ, namely increased throughput and higher quantitative accuracy, even without hipMHC normalization. Multiplexing is also relatively inexpensive because of the small amount of labeling reagent required for immunopeptidomics samples, and may also yield better fragmentation due to the formation of multiply charged ions, thereby enhancing peptide-spectrum matches for pMHC analyses.[20,38,39]

However, labeling has several drawbacks. First, sample handling steps to label peptides may result in additional sample losses, and decrease the number of peptides identified. To circumvent this limitation, repeated sampling of a given multiplexed mixture (in contrast to running the entire sample in a 'single shot' analysis) may increase the total number of quantifiable peptides,[20] although this is likely to be dependent on the number of multiplexed samples and quantity of input material. Another consideration is that multiplexed analyses will always be limited by the multiplexing capacity of existing reagents, limiting the number of samples that can be compared easily without incorporating a bridge sample.

An additional concern with isobaric multiplexing is that quantitation suffers from ratio compression to a greater extent than label-free quantification, which may obscure more subtle quantitative changes in the immunopeptidome.[40] The present authors previously tested ratio compression using titrated hipMHCs across six replicate samples, demonstrating an approximate two- to six-fold reduction in dynamic range across peptide sequences with MS^2-based quantitation.[40] This previous study also reported that inclusion of a protein carrier to 'boost' signal and thereby enhance peptide identification further increased ratio compression, diminishing the utility of this strategy for quantitative analyses. While it is possible that the use of MS^3-based quantitation[41] and/or high-field asymmetric waveform ion mobility spectrometry may improve ratio compression,[42] these approaches may reduce data quantity due to decreased sensitivity and/or slower cycle times.[38,40,43]

Ultimately, both LFQ and multiplexed methods have their unique advantages and disadvantages, and selection of the optimal approach should consider the type and number of samples that one wishes to compare quantitatively, desired quantitative accuracy, sensitivity, dynamic range, and available instrumentation and reagents. Future applications of relative quantification may include studies integrating multi-omics datasets with quantitative immunopeptidomics to better understand how the immunopeptidome is regulated by changes in cell state, as well as how the immunopeptidome shifts in response to different cell perturbations, including pathogenic conditions, therapeutic intervention, etc.

ABSOLUTE QUANTIFICATION OF PMHCS BY MASS SPECTROMETRY

Isotope dilution for single-point calibration

Multiple experimental approaches have been developed to achieve copy-level estimations of endogenous pMHCs, including the use of pMHC-specific antibodies that can be used with quantitative calibration beads and flow cytometry to estimate the number of antibody-bound pMHCs of molecules on the cell surface.[10,44,45] Unfortunately, this technique is limited to the dynamic range of the calibrants and requires a high-affinity pMHC-specific antibody for each peptide target of interest, limiting the scalability and sensitivity of antibody-based approaches for MHC quantification. Methods using targeted MS acquisition (ex. multiple reaction monitoring, selected reaction monitoring, parallel reaction monitoring) offer an attractive alternative as they can be applied to any peptide sequence, although existing approaches offer varying degrees of quantitative accuracy and difficulty of implementation.

Isotope dilution, in which a synthetic, stable-isotope-labeled (SIL) peptide standard is added to the sample, is the most straightforward strategy, with quantification occuring through comparison of the signal intensity of the endogenous (light) peptide with a synthetic peptide standard (heavy). In typical experiments, a single concentration of the heavy isotope coded standard is added (e.g. single-point calibration), and the light peptide concentration is estimated by assuming a linear dynamic range and linear relationship between ion intensity and peptide concentration (Figure 2A). These assumptions are generally valid if the signal intensities are similar for the light and heavy peptides.

There are multiple points in an immunopeptidomics workflow where standards for single-point calibration may be incorporated (Figure 2B). Most commonly, SIL peptide standards are added exogenously to a pre-purified mixture of endogenous peptides just before analysis (Figure 2B, '3') and are subsequently analysed using either discovery or targeted acquisition techniques. In 1992, Hunt et al. made the first copies-per-cell estimate of endogenous peptides by applying this technique, and estimated peptides were presented between approximately 100 and 3000 copies/cell.[2] This same technique has been employed by several authors in recent years, with measurements ranging from <10 to $>150{,}000$ copies/cell across human leukocyte antigen (HLA) class I/II and human/mouse studies.[46-48] While simple to execute, the primary limitation of this technique is that the exogenous peptide standards added prior to analysis fail to account for losses taken throughout the pMHC workflow, likely underestimating endogenous concentrations. Underestimated values may not reflect an accurate limit of detection/limit of quantitation of the experimental assay and instrumentation, and may also mislead researchers in studying the downstream consequences of different epitope abundances on the immune response.

To address this issue, several authors have added SIL peptide standards just after bound HLA pMHCs are eluted with acid (Figure 2B, '2'), prior to downstream sample processing steps such as fractionation.[49-51] Wu et al. applied this technique to quantify 21 influenza A virus epitopes spanning from 1-2 to >5000 copies/cell through direct or cross presentation, again showcasing the wide diversity in epitope abundances.[50] While this approach provides more accurate estimation of the absolute amount of pMHC expression, it does not take into account losses that may occur during IP and initial sample processing, and thus may still underestimate endogenous expression.

The extent of these sample losses was first estimated by Hassan et al. using heavy and medium SIL HLA-A2*01 peptides.[52] The authors refolded recombinant HLA alpha chain and β2m with the heavy peptides to generate heavy pMHCs

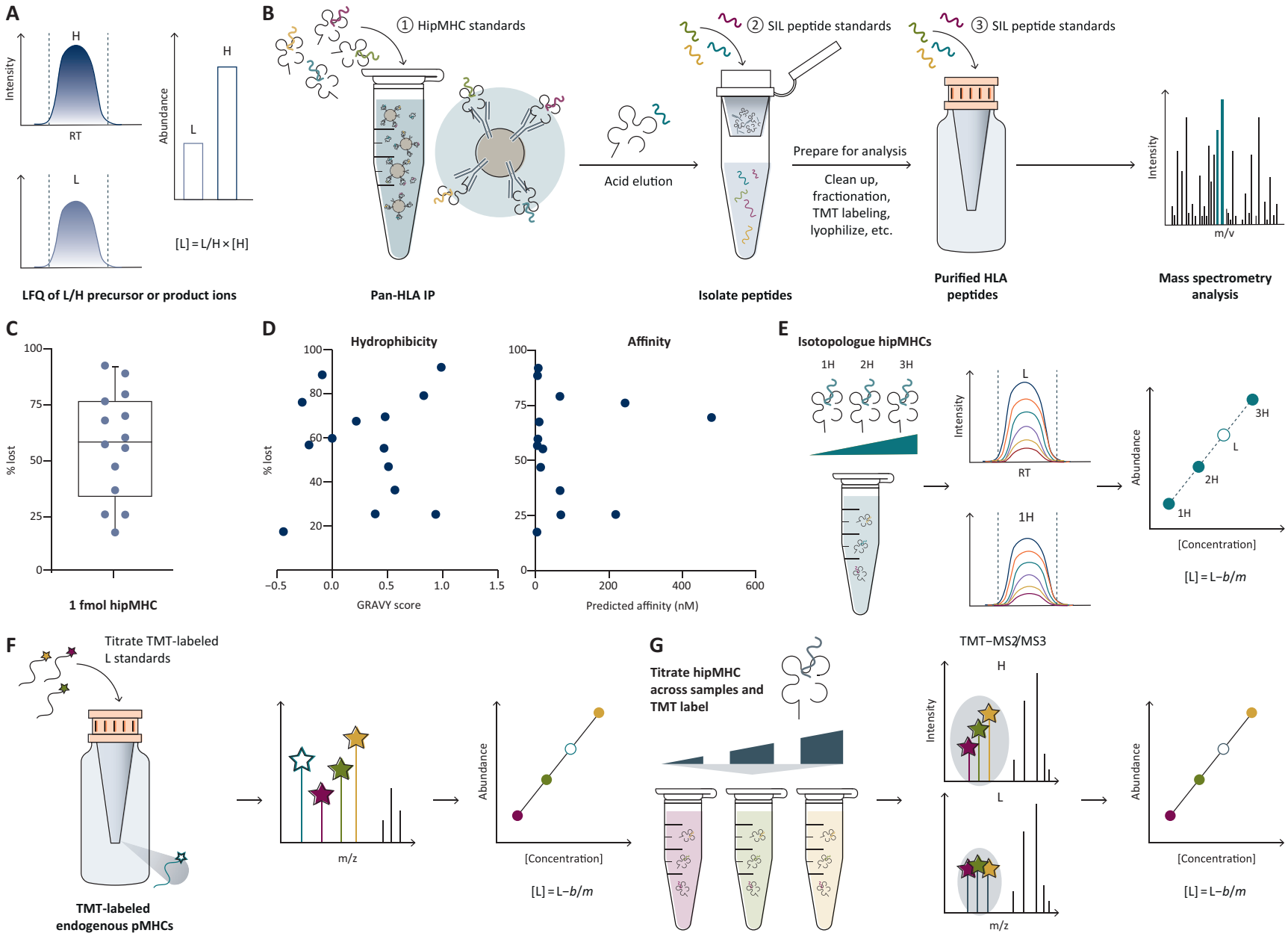

Figure 2. **(A) Absolute quantification of the light (L) endogenous peptide using single-point calibration with a heavy (H) isotopically-labeled peptide. (B) Schematic of immunopeptidomics workflow where isotopically-labeled standards may be added at points 1-3 for absolute quantification. (C) Fraction of peptide signal lost following peptide major histocompatibility complex (pMHC) enrichment and peptide isolation for $n = 14$ human leukocyte antigen-A*02:01 peptides. (D) Relationship between fraction of signal lost and hydrophobicity (GRAVY score, left) and predicted affinity (right). (E) Absolute quantification using three embedded isotopologue heavy isotopically-labeled MHC peptide (hipMHC) calibrants and SureQuant targeted data acquisition. Light concentration [L] is determined by a linear fit of the three calibrants. (F) Schematic of absolute quantification strategy using titrated, tandem mass tag (TMT)-labeled exogenous peptide standards added to a single TMT-labeled sample. [L] is calculated using multipoint TMT-labeled calibrants. (G) Schematic of absolute quantification method using hipMHC standards titrated across replicate samples and labeled with TMT prior to liquid chromatography (LC)-mass spectrometry (LC-MS)/MS analysis. [L] is calculated using the isotopic, TMT-labeled calibrants.**

LFQ, label-free quantification; SIL, stable isotope labeling.

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(hpMHCs), which were added to cell lysates prior to IP as an embedded reference. The medium-labeled peptide was added exogenously at the same concentration prior to liquid chromatography-MS/MS analysis. Using targeted MS, pMHC losses were estimated by calculating the ratio of heavy to medium peptide signal across three replicates. The results indicated striking losses during IP and sample processing, ranging from 97.2% to 99.5% for the two sequences, underscoring the need for internal pMHC standards for accurate absolute quantification. In an additional experiment, the hpMHC standard was added following complex elution, and provided estimated losses between 23% and 44%, suggesting that while the main losses occur during the IP step, losses during post-processing steps may also lead to underestimation of the endogenous concentration. To this end, Hassan et al. recommend adding hpMHCs to cell lysates prior to IP to generate the most accurate quantitation by accounting for all losses that may occur throughout the workflow (Figure 2A, '1').

To the authors' knowledge, the study by Hassan et al. represents the only estimation of sample losses in class I HLA workflows, although Wang et al. reported an experiment where recovery of class II pMHCs was estimated by spiked-in light pMHCs into cell lysate, and reported an average recovery of 74% across seven peptide sequences.[53] As HLA-IP and processing methods vary widely between researchers, the present study employed a similar strategy as Hassan et al. to estimate losses using a set of 14 HLA-A*02:01 isotopologue peptides and ultraviolet-mediated peptide exchange to generate hipMHCs.[54] While the present study used significantly fewer cells ($>10^9$ versus $<10^7$) and a lower concentration of hipMHCs (5 pmol versus 1 fmol), the present data estimates losses spanning 17.5-92% across peptides (Figure 2C, Supplementary data 1, available at https://doi.org/10.1016/j.iotech.2021.100042), with no clear relationship between sample losses and peptide hydrophobicity or predicted binding affinity (Figure 2D). While both studies failed to comprehensively capture variations in recovery that may be associated with particular alleles, sample handling techniques, cellular input quantity and pMHC concentration, these data underscore the need for internal pMHC standards added prior to IP for accurate absolute quantification. By utilizing commercially available reagents (Flex-T monomers; BioLegend, San Diego, CA, USA), hipMHC standards may be generated easily for a variety of high-frequency class I alleles.[20,25] Still, it is worth noting that many human class I and class II/mouse alleles are not commercially available at this time, which may limit the broad adoption of hipMHC-based quantitation across studies without in-house capabilities to generate the monomers of interest. Future studies assessing sample losses across a larger panel of peptides may allow for sophisticated estimation of absolute quantification without an internal calibrant, although incorporating variations associated with the peptide sequence, peptide concentration and sample processing methods is a notable challenge.

Multipoint calibration

While hipMHC standards may account for losses, single-point calibration typically assumes a linear equivalent relationship (e.g. a slope of 1) between peptide amount and ion intensity. Although the linear response range for a particular sequence can be evaluated (although time consuming, particularly with embedded single-point calibrants),[49] variations in sample input material and processing and background signal, among others, are likely to impact the linearity and slope of the intensity response across peptide sequences, even in label-free analyses.[20] As a result, absolute quantification in multiplexed experiments requires multipoint calibrants for the most accurate quantitation. Two approaches utilizing TMT to generate multiple calibration points were reported recently.[20,39] Pfammater et al. labeled a sample of interest with TMT, and titrated TMT-labeled SIL peptides into the sample prior to analysis to generate an embedded calibration curve (Figure 2F).[20,40] As the calibrants were added exogenously, this method of quantitation is likely to underestimate endogenous levels. As an alternative strategy, the present authors previously demonstrated multipoint calibration in TMT-labeled analyses by titrating hipMHCs across samples and using the TMT-labeled calibration curve of the SIL peptide to determine the endogenous concentration (Figure 2G).[39] Limitations of this approach include requiring replicate samples and difficult range-finding experiments for the calibration points due to high ratio compression in MS^2-based analyses. Future work applying this technique to MS^3-based acquisition may improve the robustness of this strategy by mitigating ratio compression, although this needs to be explored carefully.

Most recently, the present authors reported a method utilizing a series of three isotopologue hipMHCs to generate an embedded calibration curve for absolute quantification while using a fourth exogenously added isotopologue as an internal standard (IS) trigger for SureQuant IS-triggered parallel reaction monitoring (Figure 2E).[54] This sensitive and selective approach can quantify endogenous expression of pMHCs accurately at levels as low as 10 amol ($\sim$1 copy-per-cell *in vitro*), and has been applied to detect and quantify tumor-associated antigens in human melanoma biopsies. Strengths of this method include the ability to include embedded standards within a single sample, mitigating the requirement for replicate samples and avoiding any ratio compression observed with TMT-based approaches.

CONCLUSION

Changes in transcript and protein expression do not always correlate with pMHC repertoire alterations[20,35]; as such, precise molecular understanding of relative and absolute quantitative changes in pMHC expression is required to best understand how to rationally design antigen-specific targeted immunotherapies, stratify patients, and identify new antigens as therapeutic targets. For relative comparisons between a handful of samples, LFQ may be sufficient; however, for comparisons across larger cohorts, labeling

with TMT provides a useful advantage. Using embedded standards can enhance quantitative accuracy in relative quantification experiments, although researchers should be careful in interpreting both LFQ and multiplexed datasets as ratio compression may underestimate fold changes in both applications. For absolute quantification, embedded calibrants should be utilized to provide the most accurate estimates for pMHC expression, and estimates utilizing exogenous calibrants should be interpreted cautiously due to sample losses occurring during processing and analysis.

Unfortunately, one of the inherent challenges with immunopeptidomics is the variation in allelic profiles between individuals, which makes translating these techniques to clinical samples challenging. For example, without the use of an allele-specific antibody, it is challenging to compare patient samples with different allelic profiles using multiplexed methods, as the dataset would contain many missing values and would be biased towards peptides from alleles shared by multiple patients. Embedded hipMHC standards for peptides of interest can also be used to quantitatively compare relative abundance between samples, although spatial and cellular heterogeneity of patient samples can also have an adverse impact on comparisons between different tissue specimens. Improved sensitivity enabling analysis of subsets of cells from tumors could alleviate this issue, provided that cell sorting and enrichment does not alter the immunopeptidome. Advances in instrumentation and sample preparation techniques will continue to allow for improvement in data quantity and quantitative accuracy, enhancing our ability to make precise measurements within the immunopeptidome.

METHODS

Cell culture

All methods were performed as described previously.[54] Briefly, SKMEL5 cells were maintained in DMEM medium (Corning, Corning, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Amarillo, TX, USA) and 1% penicillin/streptomycin (Gibco). Cells were tested routinely for mycoplasma contamination, and maintained at 37 °C, 5% CO_2. Cells were harvested from 10-cm plates with 0.05% Trypsin-EDTA (Gibco), washed with 1X phosphate-buffered saline, and pelleted. Cells were lysed in 20 nM Tris-HCl pH 8.0, 150 mM NaCl, 0.2 mM PMSF, 1% CHAPS and 1x HALT Protease/Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA, USA) and sonicated briefly (3 x 10-s microtip pulses) to disrupt the cell membranes. Lysates were cleared by centrifugation and quantified using a bicinchoninic acid protein assay kit (Pierce, Waltham, MA, USA).

Synthetic peptide synthesis and properties

All synthetic peptides were synthesized using HeavyPeptide AQUA Custom Synthesis Service (Thermo Scientific), as described previously (Supplementary data 1, available at https://doi.org/10.1016/j.iotech.2021.100042).[54] Kyte and Doolittle GRAVY scores were calculated using Sequence Manipulation Suite v2, and predicted binding affinities were determined using pan-NetMHC 4.0[55] (Supplementary data 1, available at https://doi.org/10.1016/j.iotech.2021.100042).

Ultraviolet-mediated peptide exchange for hipMHCs

Ultraviolet-mediated peptide exchange was performed using biotinylated Flex-T HLA-A*02:01 monomers (BioLegend) as described previously,[20] with the 3H (three heavy amino acids) peptide series.[54] Concentration of stable hipMHCs following peptide exchange was assayed using the Flex-T HLA class I ELISA assay (BioLegend), as instructed by the manufacturer.

MHC isolation

Peptide MHCs were isolated from 5 mg of cell lysate by IP and size exclusion filtration, as described previously.[20] Briefly, 0.25 mg of anti-human MHC class I antibody (clone W6/32, Bio X Cell) was bound to 20 µl of FastFlow Protein A Sepharose bead slurry (GE Healthcare, Little Chafont, UK), rotated at 4 °C for 3 h. Beads were washed 2x with 20 nM Tris-HCl pH 8.0, 150 mM NaCl prior to addition of lysate and 1 fmol 3H hipMHC (Supplementary data 1, available at https://doi.org/10.1016/j.iotech.2021.100042), and incubated at 4 °C overnight. Next, the beads were washed 1x with TBS and water, and pMHCs were eluted for 20 min in 10% formic acid, rotated at room temperature. Passivated 10K MWCO filters (PALL Life Science, Port Washington, NY, USA) were used for peptide purification, and isolated peptides were stored at −80 °C. Prior to analysis, 250 fmol of each 4H trigger peptide, apart from GLFDQHFRL and AMLGTHTMEV (~2.5 pmol), and 1 fmol of each 2H peptide (Supplementary data 1, available at https://doi.org/10.1016/j.iotech.2021.100042) was added exogenously to the peptide mixture.

Mass spectrometry data acquisition and analysis

Analyses were performed on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific) coupled with an UltiMate 3000 RSLC Nano LC system (Dionex, Waltham, MA, USA), Nanospray Flex ion source (Thermo Scientific), and column oven heater (Sonation, Biberach, Germany). Peptides were loaded directly on to a 10—15-cm analytical capillary chromatography column with an integrated electrospray tip, prepared in-house (50 µm ID and 1.9 µM C18 beads, ReproSil-Pur; Dr. Maisch, Ammerbuch, Germany). The SureQuant survey analysis was performed as described previously,[54] and the SureQuant acquisition analysis method was modified so that only the 2H and 3H isotopically-labeled peptides were triggered following 4H identification. Peak areas of the six selected product ions (described previously) were extracted from Skyline Version 20.2.1.28[56] for the 2H and 3H peptides (Supplementary data 2, available at https://doi.org/10.1016/j.iotech.2021.100042), and transition areas were summed for all ions quantifiable across both peptides. The ratio of 3H to 2H signal was calculated to estimate losses during sample processing.

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DISCLOSURE

The authors have declared no conflicts of interest.

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