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**Citation:** Stopfer, Lauren E, Gajadhar, Aaron S, Patel, Bhavin, Gallien, Sebastien, Frederick, Dennie T et al. 2021. "Absolute quantification of tumor antigens using embedded MHC-I isotopologue calibrants." Proceedings of the National Academy of Sciences of the United States of America, 118 (37).

**As Published:** 10.1073/PNAS.2111173118

**Publisher:** Proceedings of the National Academy of Sciences

**Persistent URL:** <https://hdl.handle.net/1721.1/147945>

**Version:** Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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# Absolute quantification of tumor antigens using embedded MHC-I isotopologue calibrants

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Edited by K. Christopher Garcia, Stanford University, Stanford, CA, and approved August 2, 2021 (received for review June 22, 2021)

**Absolute quantification measurements (copies per cell) of peptide major histocompatibility complex (pMHC) antigens are necessary to inform targeted immunotherapy drug design; however, existing methods for absolute quantification have critical limitations. Here, we present a platform termed SureQuant-IsoMHC, utilizing a series of pMHC isotopologues and internal standard-triggered targeted mass spectrometry to generate an embedded multipoint calibration curve to determine endogenous pMHC concentrations for a panel of 18 tumor antigens. We apply SureQuant-IsoMHC to measure changes in expression of our target panel in a melanoma cell line treated with a MEK inhibitor and translate this approach to estimate antigen concentrations in melanoma tumor biopsies.**

MHC class I | immunopeptidomics | antigen presentation

Targeted immunotherapies have varying thresholds of antigen density required for an optimal antitumor immune response, and thus absolute quantification of peptide major histocompatibility complex (pMHC) antigen expression is necessary to inform appropriate therapeutic strategies (1–3). Previously, T cell receptor (TCR)-mimetic antibodies have been used to estimate copy numbers but require a specific high-affinity antibody for each target of interest, limiting broad applicability (4). Mass spectrometry-based approaches historically rely on exogenous heavy isotope-labeled peptide standards for single-point estimation (5, 6), failing to account for sample processing losses (7) and ion suppression (8).

We previously reported a technique to perform absolute quantification with an internal calibration curve, combining heavy isotope-labeled MHCs (hipMHCs) with tandem mass tags (8). While this method was successful in capturing endogenous expression of target pMHCs, substantial ion suppression limited standard curves to a 10-fold range and required replicate samples, limiting the method's ease of use and suitability for low-quantity material.

To circumvent these limitations, we developed SureQuant-IsoMHC, a method for high-sensitivity absolute quantification of MHC-I peptides from *in vitro* and *in vivo* samples. SureQuant-IsoMHC uses a series of heavy isotope-coded peptide standards (isotopologues) and SureQuant internal standard-triggered parallel reaction monitoring (IS-PRM) (9) to generate an embedded standard curve to estimate endogenous expression levels of 18 melanoma antigens. Here, we apply SureQuant-IsoMHC to profile changes in pMHC expression in a melanoma cell line with binimetinib (MEK inhibitor [MEKi]) treatment and exemplify the approach by profiling antigen levels using limited input material from human melanoma tumor punch biopsies.

## Results and Discussion

Four isotopologues were synthesized per target with an increasing number of heavy (1 to 4H) amino acids (Fig. 1A). HipMHCs were generated using the 1H, 2H, and 3H standards, quantified by an enzyme-linked immunosorbent assay (ELISA), and added to the cell lysate at a ratio of 1:10:100 to generate a multipoint calibration curve with a 100-fold dynamic range. Endogenous and isotopically

labeled pMHCs were enriched (8), and prior to analysis a high concentration of the 4H standard was added exogenously to serve as the IS trigger for SureQuant quantitation. Integrated product ion areas were summed, and a linear fit of the 1 to 3H isotopologues was used to determine the endogenous concentration.

We selected a panel of 18 pMHC targets (Iso18 panel, Dataset S1) for SureQuant-IsoMHC quantification from a multiplexed, discovery immunopeptidomics analysis of BRAF mutant melanoma SKMEL5 cells treated with binimetinib for 72 h. MEKi treatment increased surface HLA expression and resulted in dynamic changes in pMHC abundances relative to the dimethyl sulfoxide (DMSO)-treated control (Fig. 1B and C and Dataset S2A), in agreement with previous literature (10). Iso18 target peptides were predicted to bind HLA-A\*02:01, increased in presentation following MEK inhibition, and spanned a range of abundances within the immunopeptidome (Fig. 1D and Dataset S2B). This panel includes peptides derived from several well-studied tumor-associated antigens (TAAs), e.g., PMEL (gp100) and DCT (TRYP2).

To evaluate the linear intensity response of the Iso18 isotopologues against a relevant background, peptides were added exogenously at four concentrations (0.1 to 100 fmol) to a prepurified mixture of MHC peptides (Dataset S3A). For further evaluation, hipMHCs of half the panel were spiked in across five concentrations (0.1 to 1,000 fmol) to  $7.5 \times 10^6$  SKMEL5 cells (Dataset S3B). The magnitude of ratio compression within the 1- to 100-fmol titration varied from 1.75 to 9.35 $\times$ , emphasizing the need for multipoint calibrants for accurate quantitation. A sensitivity analysis showed five or more detectable transitions at 10 attomole across all peptides with one exception (KLDVGNAEV), suggesting most endogenous targets present at approximately one copy/cell should be detectable by SureQuant-based targeting (Dataset S3C).

We applied SureQuant-IsoMHC to quantify changes in expression of the Iso18 panel in SKMEL5 cells  $\pm$  MEKi, titrating 1H/2H/3H hipMHCs into  $7.5 \times 10^6$  cells (5 mg lysate) in triplicate. As expected, the 1H, 2H, 3H and endogenous (L [light]) peptides were triggered by the identification of 4H for all Iso18 peptides. For example, in the first DMSO replicate of “SLDDYNHLV,” integrated intensities approximated expected ratios, and a linear fit determined the endogenous concentration at 15.5 fmol or  $\sim$ 1,200 molecules/cell (Fig. 2A and B).

Author contributions: L.E.S., A.S.G., B.P., S.G., D.T.F., G.M.B., R.J.S., and F.M.W. designed research; L.E.S. and D.T.F. performed research; B.P. and S.G. contributed new reagents/analytic tools; L.E.S. analyzed data; and L.E.S., A.S.G., B.P., S.G., D.T.F., G.M.B., R.J.S., and F.M.W. wrote the paper.

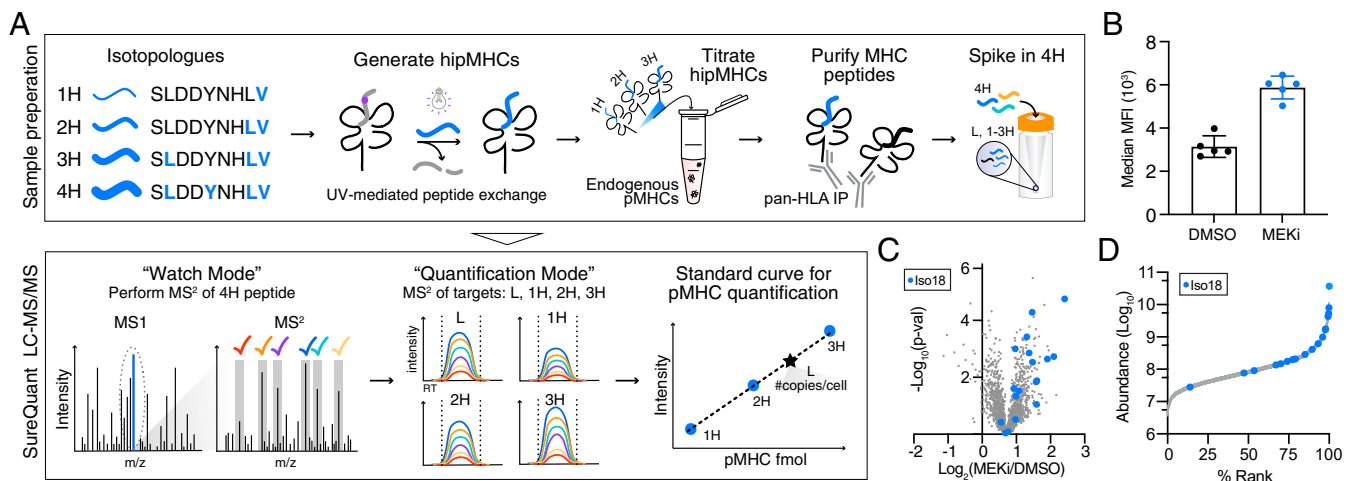
The authors declare no competing interest.

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This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2111173118/-DCSupplemental>.

Published September 8, 2021.



**Fig. 1.** Experimental workflow and peptide panel selection. (A) Schematic of SureQuant-IsoMHC sample preparation and analysis. (B) HLA-A/B/C surface expression, flow cytometry ( $n = 5$ ). (C) Mean change in surface expression for pMHCs, mean centered ( $n = 3$ ). Iso18 panel peptides are in blue. (D) Abundance of Iso18 panel peptides (blue) relative to background MHC-I peptides.

Endogenous pMHC levels spanned a wide range, and in all cases MEKi treatment increased expression (Fig. 2C). The PRUNE2-derived peptide, “GQVEIVTKV,” had an estimated 20 molecules/cell with DMSO and 250 with MEKi treatment, whereas the SLC45A2 sequence, “RLGTEFQV,” had ~40,000 molecules/cell with DMSO and ~144,000 with MEKi. SureQuant-IsoMHC provided accurate quantification across nearly four orders of magnitude, highlighting the wide diversity in expression levels of tumor antigens in the immunopeptidome.

The success of targeted immunotherapies will depend in part on the ability to confidently identify and quantify an antigen target(s) for each patient. To evaluate the levels of selected TAAs in patient tumors, we applied SureQuant-IsoMHC to 10 HLA-A2\*01<sup>+</sup> human melanoma punch biopsies to identify/quantify the Iso18 panel. A titration of 1/10/100 fmol of 1H/2H/3H hipMHC standards were added to 1.1 to 5.0 mg of homogenized tumor lysate and purified peptides were analyzed by SureQuant-IsoMHC (Fig. 2D). As tumors represent a heterogeneous composition of cell types including antigen-presenting immune cells, it is challenging to accurately discern the number of tumor cells in each punch biopsy. Therefore, we elected to express the total amount of each peptide as a fraction of the bulk sample to enable comparison across patients and to SKMEL5 cells, which similarly utilized 5 mg cell lysate.

Between 2 and 17 pMHCs were quantifiable across tumors, with only two peptides identified across the entire tumor cohort (Fig. 2E). A comparison of pMHC concentrations both across and within tumors highlight the heterogeneity of antigen presentation. For example, the BCAP31 peptide ranged from 1 to 44 fmol across tumors, whereas both NONO-derived peptides were detected below 10 fmol in all instances. Perhaps most striking are the eight peptides that were only identified in four or fewer tumors, particularly those derived from common TAAs like “SLADTNSLAVV,” a PMEL peptide identified in just three tumors. As all tumors were HLA-A2\*01<sup>+</sup>, these data showcase the diversity of endogenous pMHC presentation among tumors even with a common allele. The interpatient heterogeneity revealed by our analysis points to the need for targeted assays like SureQuant-IsoMHC to verify and quantify expression of antigens used in targeted immunotherapies, which may serve to better stratify eligible patients and enhance personalized therapeutic approaches.

To assess the sensitivity of SureQuant-IsoMHC in clinical samples, we performed data-dependent acquisition (DDA) on another aliquot of the isolated tumor peptides to determine whether the Iso18 targets

could have been identified in discovery-mode workflows (Dataset S4). While eight Iso18 targets were identified in discovery mode in T1 (Fig. 2F), most tumors had between zero and two Iso18 target identifications (Fig. 2G). Indeed, most peptides, quantified below 1 fmol with SureQuant-IsoMHC, were not identified in the discovery analyses (Fig. 2F), confirming the bias of DDA toward higher abundant epitopes. Moreover, T8, which used just 1.1 mg of tumor lysate input, had zero Iso18 identifications with DDA and seven with SureQuant-IsoMHC, highlighting the method’s sensitivity in detecting and quantifying low-abundance epitopes.

As there are many antigen-specific targeted immunotherapies in clinical development, verifying the presence and concentration of target antigens in small quantities of patient tumor specimens is of increasing importance. SureQuant-IsoMHC provides a high sensitivity, highly reproducible solution for the accurate quantification of even low-abundance target antigens. Here we targeted well-characterized tumor antigens; however, this method may be similarly leveraged for predicted neoantigens or viral epitopes using minimal tumor material. These lowly abundant targets have historically been challenging to identify using DDA, even with large amounts of sample (11), rendering SureQuant-IsoMHC an attractive solution.

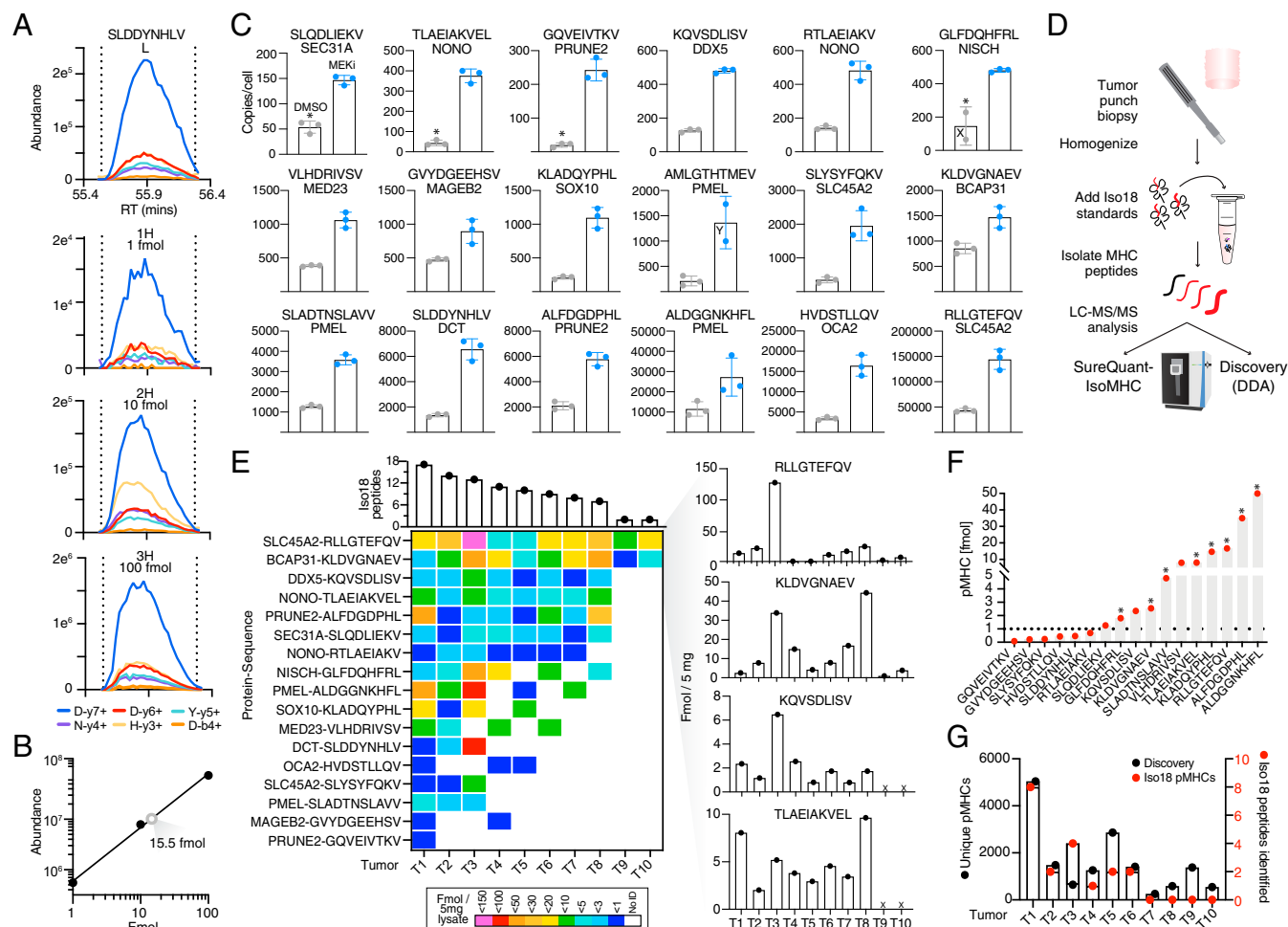
Future studies may utilize SureQuant-IsoMHC to characterize the expression levels of known or predicted antigens across a larger tumor cohort and expand beyond HLA-A\*02:01. These data may be used to better elucidate the relationship between antigen expression and other biomarker measurements (i.e., transcript/protein expression), which could prove beneficial in defining which patients may benefit from specific immunotherapies.

## Materials and Methods

Detailed descriptions are provided in *SI Appendix*.

Patients with metastatic melanoma at Massachusetts General Hospital (Boston, MA) provided written informed consent for the collection of tissue and blood samples for research and genomic profiling. This study was approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board (DF/HCC Protocol 11-181). Tumor samples were collected, snap frozen, and stored at  $-80^{\circ}\text{C}$  prior to analysis.

hipMHCs of isotopologues were generated using ultraviolet (UV)-mediated peptide, quantified by ELISA, and added into SKMEL5 cell and tumor lysates. Heavy and endogenous pMHCs were purified by immunoprecipitation using a pan-specific HLA class I antibody (w6/32), and peptides were isolated by size exclusion filtration as previously described (8). The 4H trigger peptide was added exogenously, and peptides were subsequently analyzed using the



**Fig. 2.** SureQuant-IsoMHC quantification in vitro and in vivo. (A) Product ion traces for “SLDDYNHLV.” (B) Linear fit of summed intensities ( $r^2 = 0.997$ ) and interpolation of endogenous concentration for “SLDDYNHLV” with DMSO. (C) Copies-per-cell estimates of pMHCs with DMSO/100 nM MEKi,  $n = 3$  except  $n = 2$  where “X” = L below limit of detection and “Y” = 4H below intensity threshold. Error bars  $\pm$  SD; \*extrapolated. (D) Tumor analysis workflow. (E) Peptide concentration per 5 mg lysate. (F) T1 Iso18 concentrations. Dotted line is lowest calibration point, \*pMHCs identified with DDA. (G) Number of unique peptides (Left, black) and Iso18 panel peptides (Right, red) identified with DDA.

SureQuant IS-PRM acquisition framework on an Exploris 480 mass spectrometer. Data were analyzed using Skyline software (12).

**Data Availability.** Mass spectrometry data files have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD024917. Dataset S7 contains a filemap. All other study data are included in the article and/or SI Appendix.

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**ACKNOWLEDGMENTS.** We thank Andreas Huhmer at Thermo Fisher Scientific for funding the synthetic peptide standards, and Eric Berg for peptide synthesis support. This research was supported in part by NIH grants U54CA210180 and U01CA238720, as well as funding from the Melanoma Research Alliance (MRA Team Science Award 565436) and the Massachusetts Institute of Technology Center for Precision Cancer Medicine. L.E.S. is supported by a NIH training grant in Environmental Toxicology (T32-ES007020).

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