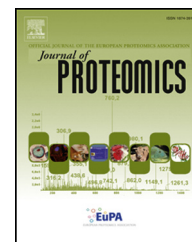


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Technical note

Accurate quantitation of MHC-bound peptides by application of isotopically labeled peptide MHC complexes

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ABSTRACT

Knowledge of the accurate copy number of HLA class I presented ligands is important in fundamental and clinical immunology. Currently, the best copy number determinations are based on mass spectrometry, employing single reaction monitoring (SRM) in combination with a known amount of isotopically labeled peptide. The major drawback of this approach is that the losses during sample pretreatment, i.e. immunopurification and filtration steps, are not well defined and must, therefore, be estimated. In addition, such losses can vary for individual peptides. Therefore, we developed a new approach in which isotopically labeled peptide-MHC monomers (hpMHC) are prepared and added directly after cell lysis, i.e. before the usual sample processing. Using this approach, all losses during sample processing can be accounted for and allows accurate determination of specific MHC class I-presented ligands. Our study pinpoints the immunopurification step as the origin of the rather extreme losses during sample pretreatment and offers a solution to account for these losses. Obviously, this has important implications for accurate HLA-ligand quantitation. The strategy presented here can be used to obtain a reliable view of epitope copy number and thus allows improvement of vaccine design and strategies for immunotherapy.

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Abbreviations: AQUA, absolute quantitation; EBV-LCL, Epstein-Barr virus transformed B Lymphoblastic cell line; IP, immunoaffinity purification; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; MiHA, minor histocompatibility antigens; PE, phycoerythrin; pMHC, peptide MHC class I complex; hpMHC, pMHC loaded with heavy isotope labeled peptide; SRM, selected reaction monitoring.

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Peptides presented by human leukocyte antigen (HLA) molecules on the cell surface play a crucial role in immunology, and mediate the communication between T cells and antigen presenting cells. Both the identity and abundance of these peptides are of great importance in fundamental studies on T cell action and in design of T cell-mediated therapy. These include tumor immunotherapy [1] and treatment of hematological malignancies by a combination of hematopoietic stem cell transplantation (HSCT) and treatment with donor T cells specific for minor histocompatibility antigens (MiHA) expressed on malignant cells from the patient [2]. Knowledge of accurate copy number of MHC class I presented epitopes can for example be used to study the relationship between differing epitope abundances and T cell immunodominance hierarchies [3,4], and thereby help to improve vaccination strategies to elicit more effective immune responses. In addition, knowledge of ligand copy number is important in fundamental studies on antigen processing.

Currently, the best copy number determinations are based on mass spectrometry, employing SRM in combination with mixing in a known amount of isotopically labeled peptide, known as the AQUA approach [3–6]. The AQUA standard can be mixed in at different stages during sample pretreatment, but preferably as early as possible. The complexity of the ligandome [7] demands various sample pretreatment steps before the actual peptide quantitation by LC-MS, involving cell lysis, immunoaffinity purification (IP), filtration and usually chromatographic fractionation. The major uncertainty in the accurate quantitation of ligand copy number is not caused by the final mass spectrometric experiment, but introduced by the variable yields of the sample pretreatment steps, which in addition can be peptide dependent. However, the overall yield of the sample pretreatment was not determined in previous studies, but rather estimated.

Therefore, we developed a new approach extending the AQUA approach in which isotopically labeled peptide-MHC monomers, i.e. peptide-MHC monomers loaded with a peptide in which two heavy amino acids have been incorporated (heavy peptide-MHC, or hpMHC), were prepared as a spike in standard and added directly following cell lysis, after which the usual sample processing was applied. Using this approach all losses during sample processing, including those during immunopurification, can be accounted for and it allows accurate determination of specific MHC class I-presented ligands, see Fig. 1. We added a medium labeled peptide just before final LC-SRM analysis to enable determination of the overall yield of the sample pretreatment. Our hpMHC approach (addition of hpMHC before IP) was compared with the AQUA approach (addition of heavy peptide after IP).

T cells specific for MiHA have been shown to induce graft versus leukemia reactivity after allogeneic stem cell transplantation [2,8,9], and a number of MiHA has been used to design strategies for cellular immunotherapy but with limited success [10,11]. An accurate quantitation of the presented peptides involved may help to understand the efficacy of this treatment. Here, we illustrate our approach by analysis of two recently discovered minor histocompatibility antigens (MiHA), presented in the HLA-A*0201 molecule called (LB-NISCH-1A, ALAPAPAEV) [12], and LB-SSR1-1S, VLFRGGPRGSLAVA [13]. To assess the copy number of the endogenous (light) MiHA, we added the

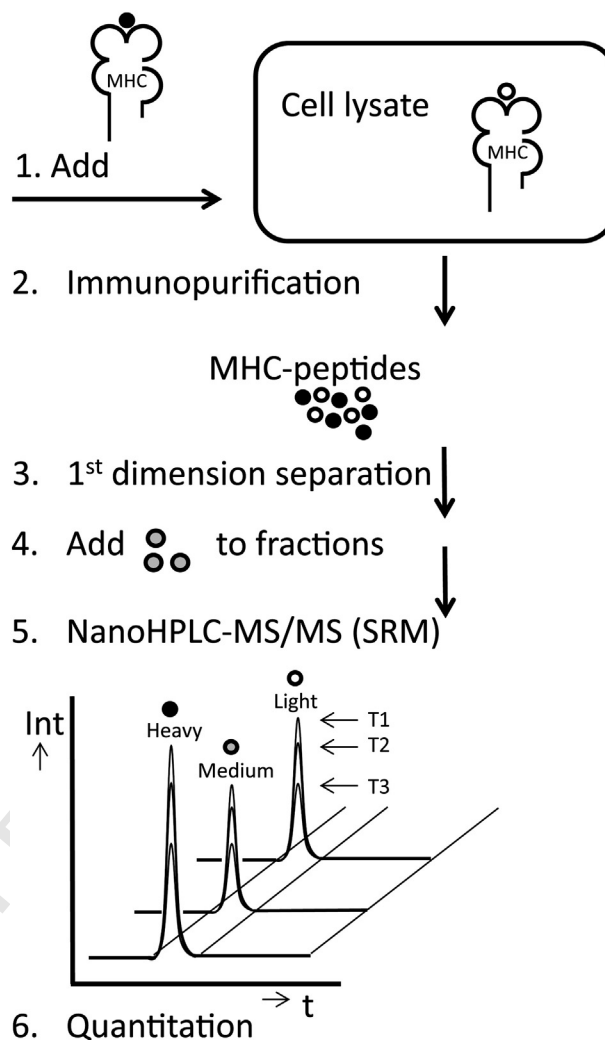


Fig. 1 – Strategy for the accurate quantitation of MHC-class I-presented peptides. An MHC-monomer loaded with heavy labeled peptide (hpMHC) is added to the cell lysate (step 1) in which the endogenous (wt; unlabeled) peptide is present. Subsequently, the hpMHC and the wt-pMHC undergo the same further sample pretreatment. Just before LC-MS analysis a medium labeled variant of the peptide is added (step 4) for determination of the yield of the procedure. Black dots: heavy peptide, gray dots: medium peptide and open dots: wt (unlabeled) peptide. The three different forms of the peptide, light, medium and heavy, coelute and these three forms are all quantitated by 3 SRM-transitions (T1-T3).

HLA-A*0201-monomer loaded with stable heavy isotope labeled peptide ALAPAPAEV or VLFRGGPRGSLAVA ($\Delta M = +13$ Da) as internal reference (heavy isotope labeled amino acids are in bold and underlined). The hpMHC were prepared by folding of recombinant HLA alpha chain and $\beta 2m$ in the presence of the heavy isotope labeled peptide. We combined the hpMHC with the AQUA strategy by using the medium labeled peptide ALAPAPAEV or VLFRGGPRGSLAVA ($\Delta M = +7$ Da), which was added to the chromatographic fractions just before final LC-MS quantitation. The medium labeled peptide for AQUA was added to each fraction prior to LC-SRM (or PRM) analysis to determine

the overall yield of the sample pretreatment procedure. In the AQUA approach the labeled peptide is preferably added as early as possible during sample pretreatment. In contrast, we have added the medium peptide just before MS analysis to be able to calculate the overall yield of the hpMHC procedure, i.e. the ratio of the medium and heavy labeled peptide.

The quality of the hpMHC was assessed by mixing equimolar amounts of hpMHC and the medium labeled peptide. As can be seen in Supplemental Figure S1, the medium and heavy peptides display the same peak height in the MS spectrum, from which we conclude that the purified hpMHC has been correctly loaded with a single peptide, and the measured hpMHC concentration, as determined by Bradford, is in line with the pure synthetic peptide concentration. Therefore, the intensities of the heavy peptide and the medium peptide can be used for their relative quantitation.

Initial LC-SRM measurements (bioreplicate 1) were performed on a Q-TRAP instrument. Similar SRM transitions were used per peptide for the light, medium and heavy labeled peptides (three transitions per peptide). After collision energy optimization, the most abundant transitions were chosen, as displayed in Supplemental Table S1, and Supplemental Figure S2 for the MS2 spectra. Bioreplicates 2 and 3 were recorded by PRM on a Q-Exactive.

Next we prepared a calibration curve, in which the linearity of the mass spectrometric determination of the medium labeled AQUA peptides ALAPAPAEV and VLFRRGGPRGSLAVA were verified with the pure synthetic peptides dissolved in buffer A and verified with the pure synthetic peptides dissolved in matrix. The matrix was composed of a pool of neighboring first dimension C18 HPLC fractions, not containing the peptide of interest. The response was found to be linear between injections of 100 amol

to 100 fmol absolute on column, see Supplemental Figure S3. Following the completion of the calibration curve, the MiHA LB-NISCH-1A and LB-SSR1-1S were quantitated. To this end, the cell lysate of 6.7×10^9 (6.7E9) EBV-LCL JYpp65 cells was spiked with 10 pmol of each hpMHC for bioreplicate 1. For bioreplicates 2 and 3, 2.0×10^9 (2E9) cells were spiked with 5 pmol of each hpMHC. After immunopurification of HLA class-I molecules, the HLA-class I α -chain and β 2m proteins were separated from the peptides by 10 kDa filtration and the peptides were fractionated using C18 chromatography and freeze dried. Just before mass spectrometric quantitation, fractions were redissolved in solvent A, supplemented with the medium labeled peptides to a final concentration of 2.5 and 3.5 fmol/ μ l for ALAPAPAEV and VLFRRGGPRGSLAVA, respectively.

The results are listed in Table 1 and shown in supplemental Figure S4. The spiked-in medium labeled peptide is present in all fractions. The ratio between the three SRM transitions of each MicbHHA is constant, so there is no interference from other potentially contaminating background peptides. The heavy labeled peptide and the light (endogenous) peptide, which both underwent the whole sample pretreatment procedure, are coeluting as expected and are only present in two neighboring fractions. The overall yield of the sample pretreatment was calculated from the ratio of the medium labeled peptide and the heavy labeled peptide. As an example for bioreplicate 1, for LB-NISCH-1A the sum of the heavy labeled peptide peaks in fractions 16 and 17 (panel C) should correspond to 330 fmol of heavy peptide (i.e. 1/30th of 10 pmol spike in of hpMHC in lysate, since 1/30th of the peptide pool was injected on column) at 100% peptide recovery from the

Table 1 – Quantitation summary of the native LB-NISCH-1A and LB-SSR1-1S peptides for the 3 biological replicates. Total peptide amounts, recovery and copy numbers are listed. For the AQUA measurements no copy number is listed, since the yield is not known. For the AQUA experiments the amount of heavy peptide and recovery is listed. n.a. not applicable.

hpMHC-approach			
LB-NISCH-1A (ALAPAPAEV)	Native peptide (pmol)	Recovery	Copy number
Sample 1 (6.7E9 cells)	8.8 $\pm$ 0.1%	1.5%	789 $\pm$ 7
Sample 2 (2E9 cells)	17.6 $\pm$ 7.1%	0.9%	5273 $\pm$ 374
Sample 3 (2E9 cells)	10.0 $\pm$ 3.6%	1.1%	3003 $\pm$ 108
LB-SSR1-1S (VLFRRGGPRGSLAVA)	Native peptide (pmol)	Recovery	Copy number*
Sample 1 (6.7E9 cells)	33 $\pm$ 0.4%	2.8%	2943 $\pm$ 36
Sample 2 (2E9 cells)	33.2 $\pm$ 6.7%	0.8%	9960 $\pm$ 667
Sample 3 (2E9 cells)	40.1 $\pm$ 3.2%	0.5%	12,030 $\pm$ 385
AQUA-approach			
LB-NISCH-1A (ALAPAPAEV)	Heavy peptide (pmol)	Recovery	Copy number*
Sample 1 (6.7E9 cells)	n.a.	n.a.	n.a.
Sample 2 (2E9 cells)	1.5 $\pm$ 7.4%	29.4%	n.a.
Sample 3 (2E9 cells)	1.3 $\pm$ 3.9%	25.8%	n.a.
LB-SSR1-1S (VLFRRGGPRGSLAVA)	Heavy peptide (pmol)	Recovery	Copy number*
Sample 1 (6.7E9 cells)	n.a.	n.a.	n.a.
Sample 2 (2E9 cells)	2.2 $\pm$ 11.7%	43.5%	n.a.
Sample 3 (2E9 cells)	1.2 $\pm$ 2.8%	23.3%	n.a.

lysate. However, the actual response corresponds to approximately 4.9 fmol (the sum of the medium labeled peptides in fractions 16 and 17 in panel A). Therefore, the yield of the heavy labeled LB-NISCH-1A peptide, derived from the hpMHC, is determined to be 1.5% (4.9/330). The amount of endogenous LB-NISCH-1A (panel B) can easily be calculated from the ratio of the heavy peptide and light peptide, and was determined to be 8.8 pmol. The absolute amount of 8.8 pmol on 6.7E9 cells corresponds to a copy number of 789, see Table 1. Similarly for LB-SSR1-1S the yield of the heavy labeled peptide (panel F) was determined to be 2.8%. The amount of endogenous LB-SSR1-1S-1A (panel E) was calculated to be 33 pmol, which corresponds to a copy number of 2943, see Table 1. Similarly the numbers for the other bioreplicates were determined. These numbers represent the cell surface copy number, since the majority of the pMHC is on the cell surface, rather than in an internal compartment, see Figure S5.

Striking differences in recovery were observed between the hpMHC and AQUA approach. The steps following the IP, comprising filtration, first dimension chromatographic fractionation and the final LC–MS run, have an overall recovery of 23 to 43% (which amounts to 3 steps of approximately 70% on average per step), as determined by the AQUA approach. In sharp contrast with the former the yield of the hpMHC approach was determined to be 0.5–3%. Therefore, rather extreme losses, i.e. 90–99%, occur during the immunopurification step.

Quite some variations in native peptide copy number between biological replicates can be observed in Table 1. It is important to note that the ratio between the medium peptide and the heavy peptide (added as hpMHC) was constant throughout all experiments, as it should since these were taken from the same stock solution for all experiments. The former rules out any issues with the added labeled peptide and hpMHC. Whether the measured variation can be fully attributed to biological variation is not clear. The stability of the native pMHC complexes during cell storage might play a role.

The hpMHC approach was used to account for all losses during MHC ligand isolation and purification. Our results show that the use of hpMHC allows accurate quantitation of the HLA class I-presented peptides, by accounting for all losses during sample pretreatment. These losses can be very serious, as shown for the MiHA presented in this study. Since our first elution experiments in the mid 1990s we have found these low yields. The fact is that the average yields are indeed low, which we illustrated to be approximately 5% on the basis of total HLA measured versus total peptide intensity in the mass spectrometer [7]. Based on the current measurements, with a lower starting number of cells, the average yield seems closer to 1–2%. In addition, a simple calculation in combination with final mass spectrometric responses shows the same. Starting from 1 billion cells (1E9) and assuming a conservative number of 300.000 HLA I molecules on the cell surface of an EBV-LCL, we calculate a total number of 3E14 HLA I molecules. Using Avogadro's number this yields (3E14/6E23 = 0.5E–9 mol), i.e. 500 pmol HLA. Assuming 1000 different ligands representing the majority of the total HLA-ligandome, the amount per peptide totals 500 fmol. These peptide levels, based on 100% yield, are not observed in our nanoHPLC-experiments. We detect much lower amounts, compared to known peptide standard responses. Furthermore, we have also performed MHC-elutions, according to Illing et al. [14] of

as low as 25 million cells, still identifying over 1100 HLA ligands using the same stringent criteria used in Hassan et al. [7]. These 1100 identified ligands represent a very competitive number, so the losses we report and compensate for, appear to be real and seem a general phenomenon in peptidome analysis. Summarizing the above, we conclude that sample loss issues have never been fully addressed analytically. We are confident that the pMHC monomers are stable, since we can fold stable pMHC monomers and subsequently stable tetramers for T cell staining. All of the above, to our opinion, clearly illustrates the need for an internal standard from the beginning of the sample processing, i.e. the need for a hpMHC. Naturally, for every peptide of interest the corresponding hpMHC must be prepared, similar to LC-SRM drug/metabolite monitoring experiments of small compounds in which specific heavy isotopes are introduced by chemical synthesis. Similarly, this strategy is applicable to MHC class II complexes, although these cannot yet as easily be produced as class I pMHC complexes. The strategy presented here can be used to get a better view of epitope copy number and as such improve vaccine design and immunotherapy.

Conflict of interest

The authors have declared no conflict of interest.

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