Online Supplemental Material for

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Quantitation and Reproducibility of Peptide Repertoire Analysis Using MALDI-TOF

We have taken extreme care to ensure maximal reproducibility of the MALDI spectral analysis of the peptide fractions eluted from both cell lines. This necessarily involved measures at all steps of the purification. We used cells with similar levels of class I expression as determined by an α-Bw4 mAb and compared eluates generated from comparable amounts of recovered class I protein as determined by staining intensity of coomassie blue stained gels of retained protein after the ultrafiltration step. The eluates from the two cell lines were prepared in parallel using identical affinity matrices. RP-HPLC separation has been optimized in the laboratory, column temperature is monitored and kept as constant as possible, and we use an instrument with extremely reproducible gradient delivery. The same column is used for each eluate with thorough cleaning steps in between runs.

From each separation, ~60 fractions are analyzed by MALDI-TOF. An aliquot of the fraction (0.5–1 μl) was mixed with an equal volume of matrix solution in a microtube to ensure uniform distribution of the peptide in the matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in acetonitrile/ethanol 1:1 vol/vol), spotted onto a target, and dried for analysis. This ensures a uniform distribution of cocrystals of sample and matrix. Great care is taken during data generation to ensure a representative dataset is collected. Replicate analysis has been performed on all fractions, with essentially the same result. In addition, the entire elution experiment (i.e., cell culture, immunoaffinity purification, and elution) was performed on several occasions, yielding similar results. Although some variation in the retention time (<1%) was observed for some of the species, resulting in their falling into neighboring fractions relative to the other analyses, all those species observed to be shared or preferentially presented by one allele and not the other were consistent between elution experiments (Fig. S1). We have also demonstrated this reproducibility with elution from other class I alleles including H-2K^b (1) and HLA-B*2705 (2).

Although no external standard is added to the sample for internal quantitative analysis, many of the species are shared by HLA B*4402 and B*4403; thus, we argue that relative changes in signal intensity between shared and unique peaks highlight repertoire differences. This is shown clearly in Fig. S2.

Of note, the 1,145.3 amu peak is present in both eluates and is detected at similar intensity in both samples as is the species at 1,242.4 amu. In striking contrast the species at 1,121.4 amu is far more abundant in the B*4403 eluate (i.e., spectra of negative polarity). As such, the 1,145.3 and 1,242.4 amu species act as internal calibrants, and we feel confident that we can say that the species at 1,121.4 amu is preferentially found in the HLA-B*4403 eluate. Thus, although absolute intensity of species observed in different samples is a poor comparator, relative intensity to these internal landmark species gives relative quantitation. This is based on the caveat that all other things are equal (i.e., they are both ionized from complex mixtures that contain many of the same species, and individual peaks compared represent the same amino acid sequence, removing any bias in ionization, solubility in the matrix, and proton mobility). We have recently reviewed the comparative use of MALDI-TOF and ESI-Qq-TOF for immunological studies (3).

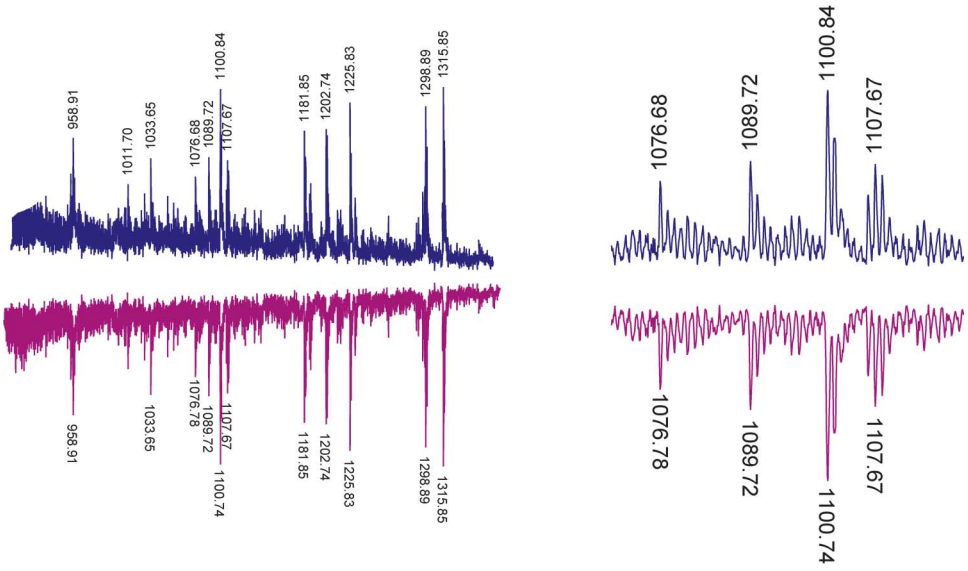

Figure S1. Analyses of fractions derived from two independent elution experiments. Replicate analysis reveals highly reproducible fraction content and high mass accuracy. Exploded region of spectra revealing 10 ppm mass accuracy and isotopic resolution of individual species.

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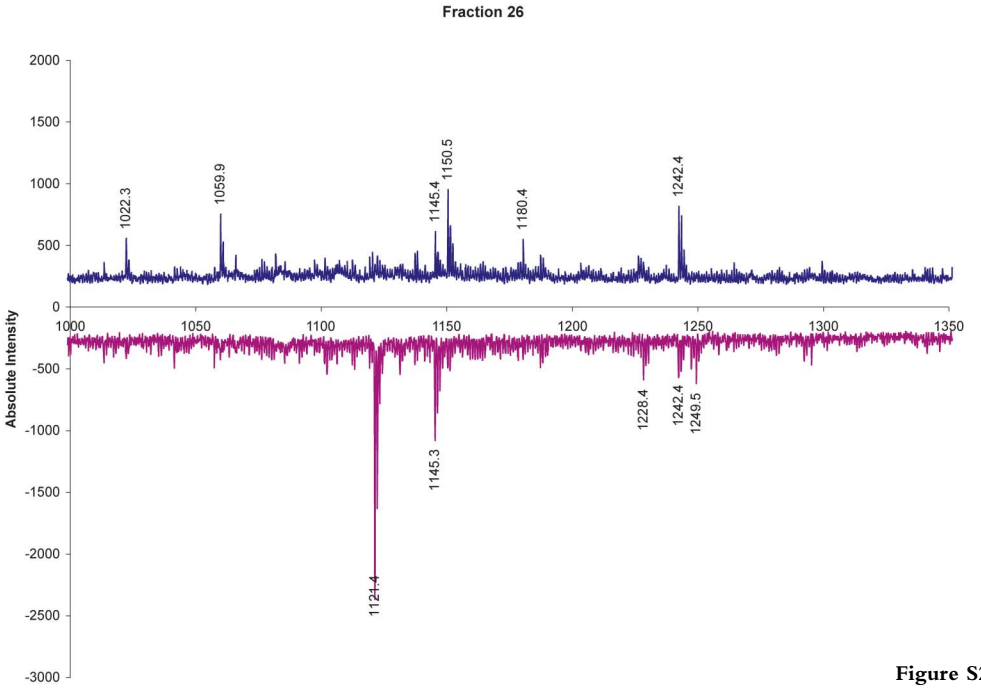

Figure S2.

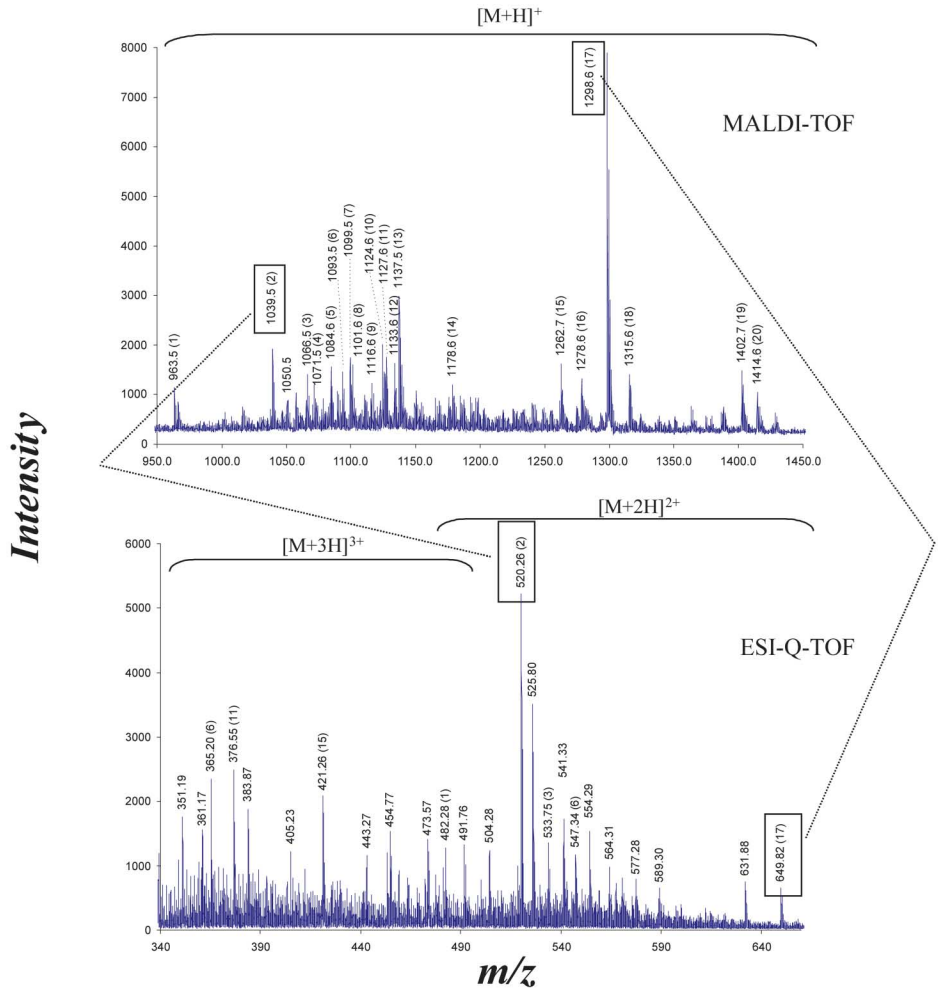

Figure S3. MALDI-TOF and ESI-Qq-TOF analysis of fractionated peptides derived from HLA B*4403 eluates reveal differences in the representation of specific peptide species. MALDI-TOF mass spectrometry was performed using a Reflex mass spectrometer (Bruker-Franzen Analytik) operated exclusively in the reflectron mode as described previously (4). An aliquot of a fraction (1–2 μl) containing peptides isolated from HLA B*4403-expressing C1R cells was mixed with an equal volume of α-cyano-4-hydroxycinnamic acid (10 mg/ml in acetonitrile/ethanol 1:1 vol/vol), spotted onto a target, and dried for analysis. ESI-Qq-TOF mass spectrometry was performed using a Q-STAR mass spectrometer (PE-SCIEX; PE Biosystems) equipped with a Protana nanospray source. The twenty most abundant species in the MALDI-TOF MS profiles of this fraction are also highlighted in the ESI-Q-TOF MS spectra. This figure was reproduced from (3).

Quantitation of individual peptides from biological matrices is a difficult issue in that no form of mass spectrometry offers a perfect solution. The use of internal standards and isotope tagging provides quantitative data but in mixtures as complex as those derived from HLA-bound peptides, these techniques become more difficult and signals from the calibrants may mask authentic peptide signals. Thus, we have chosen to use shared natural ligands as the benchmark for relative quantitation rather than an external standard to provide absolute quantitation.

Moreover, the form of ionization also effects the quantitative information contained within the spectra, with both ESI and MALDI having advantages over the other. ESI clearly has a closer relationship between peptide concentration and signal intensity; however, this quantitative nature is influenced and hampered by various factors as follows: (a) ESI is more sensitive to minor contaminating species (e.g., salts, detergents, etc.) that effect ionization efficiency; (b) ESI is less amenable to automation and requires expert users, whereas MALDI analysis can be automated to reduce systematic errors; (c) ESI spectra of this type tend to have more chemical noise, making rarer species less easily distinguished in the spectra. In our experience, a 10-fold decrease in signal to noise is typical with these levels and types of analytes; (d) Multiple-charge states exist for peptides in ESI (+2 and +3 are frequently observed for MHC class I peptides using our Qq-TOF instrument), whereas these multiple-charged species are useful for sequencing the multiplicity of peaks observed for individual species complicates the spectra, making it difficult to identify individual components and quantitate them. The formation and relative proportions of +2:+3 varies with peptide sequence and a manner that is not easily predicted (Fig. S3); (e) Additional adducts are found more frequently in ESI, such as sodiated species and potassium adducts.

It should also be noted (Fig. S3) that abundant species present in the MALDI-TOF spectra are also observed with good signal in the ESI-Q-TOF spectra. Moreover, although the relative intensities of individual species may change between modes of ionization, the relative intensities for these species observed in B*4402-derived or B*4403-derived fractions by the same mode of mass spectrometry remain the same, except for those species observed to be preferentially bound to one allele and not the other. In this case, these species are reduced in both modes of mass spectrometry.

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