HLA Antibody-Based Immunoprecipitation Protocol for Immunopeptidomics

Protocol description:

The protocol isolates HLA (HLA-I or HLA-II) using antibody bound to sepharose beads. In this protocol, HLA peptide complexes are eluted off the beads with acid, desalted and analyzed by mass spectrometry. This allows for the direct detection of potential antigen targets of T cell responses.

Sample requirements

Sample types:

- Flash-frozen tumors/tissue (low ischemic times <1 hr)
- Flash-frozen cell lines (washed 3x with PBS)
    - We do not prefer viable cells, as the DMSO can negatively impact our protocol
- OCT scrolls (not ideal, but we can handle them)

Typical input requirements:

- 10-50 mg wet wet tissues
- 10 million cells (we can go as low as 1 million, but the data depth will suffer)

Sample metadata requirements:

- High resolution HLA typing
- Tumor purity
- Details on sample collection protocols and if any sorting or enrichment was used
- If both HLA-I and HLA-II are requested, we require flow or RNA-seq data to support the expression of both HLA-I and HLA-II

Serial HLA-II and HLA-I peptide complex enrichment and peptide acid elution

Eppendorf tubes were pre-conditioned by rinsing twice with 1 mL high-performance liquid chromatography (HPLC) water, incubating overnight with 1 mL HPLC water, and then rinsing twice more with 1 mL HPLC water. Cryopulverized samples were thawed on ice (20 minutes), lysed in 1.2 mL of lysis buffer at 4°C (20 mM Tris-HCl pH 7.5 (Invitrogen, Waltham, Massachusetts, USA, 15567027), 1 mM Ethylenediaminetetraacetic (EDTA) (Invitrogen, Waltham, Massachusetts, USA, 15575-038), 100 mM NaCl (Sigma-Aldrich, St. Louis, Missouri, USA, 71386-1L), 6 mM MgCl2 (Sigma-Aldrich, St. Louis, Missouri, USA, 63069-100ML), 60 mM Octyl β-d-glucopyranoside (Sigma-Aldrich, St. Louis, Missouri, USA, O8001-25G), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, Missouri, USA, 93482-250ML-F), 0.2 mM Iodoacetamide (Thermo Fisher Scientific, Waltham, Massachusetts, USA, A39271),

1.50% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA, T9284-500ML), 1x C0mplete protease inhibitor tablet-EDTA free (Sigma-Aldrich, St. Louis, Missouri, USA, 11873580001), 10 mM NaF (Sigma-Aldrich, St. Louis, Missouri, USA, S7920), 1:100 dilution Phosphatase Inhibitor Cocktail II (Sigma-Aldrich, St. Louis, Missouri, USA, P0044), 1:100 dilution Phosphatase Inhibitor Cocktail III (Sigma-Aldrich, St. Louis, Missouri, USA, P5726), 110 mM Sodium Butyrate (Sigma, B5887), 2 µM suberoylanilide hydroxamic acid (SAHA)(Sigma,SML0061), 10 mM nicotinamide (Sigma, N3376), and 50 µM PR-619 (Lifesensors, SI9619: PR-619) in pre-conditioned 0.6 mL Eppendorf tubes, and incubated for 30 min with 2 µL benzonase (Sigma-Aldrich, St. Louis, Missouri, USA, E1014-25KU). After incubating in lysis buffer, the lysates were centrifuged at 15,000 rcf for 20 min at 4°C.

To pre-clear the samples, the supernatants were transferred to another set of pre-conditioned 1.5 mL Eppendorf tubes containing Gammabind Plus Sepharose beads (Sigma-Aldrich, St. Louis, Missouri, USA, GE17-0886-01) that were washed 3 x with 1 mL 4°C PBS and incubated with end-over-end rotation at 4°C for 1 hr. Gammabind Plus Sepharose beads were pelleted at 1500 rcf for 1 min at 4°C. The pre-cleared supernatants were transferred to a new pre-conditioned 1.5 mL Eppendorf tube for the subsequent, serial HLA-II and HLA-I enrichment steps.

Immunoprecipitation (IP) of HLA-II and HLA-I peptide complexes were performed sequentially as follows: For HLA-II peptide complex IP, ~37.5 µl PBS washed gamma bind sepharose beads (MilliporeSigma; catalog no.: GE17-0886-01) and 15 µg of HLA-II antibody mix (9 µg TAL-1B5 [Abcam; catalog no.: ab20181]), 3 µg EPR11226 (Abcam; catalog no.: ab157210), and 3 µg B-K27 (Abcam; catalog no.: ab47342) were added to pre-cleared lysates in a 2 mL deepwell plate(Cytiva, 7701-5200), and plates were sealed with 96-well square sealing mats (Thermo Fisher Scientific; catalog no.: AB0675). HLA-peptide complexes were captured on the beads by incubating end-over-end on a rotor at 4 °C for 3 h. Following the incubation beads and lysates were transferred to a prewashed 10 µm PE fritted plate (Agilent; catalog no.: S7898A) stacked on top of a fresh 96 well, 2 ml deep plate. Beads with HLA-II peptides complexes were retained on the filter plate, whereas lysates were collected in the fresh 2 ml 96-well plate. For HLA-I enrichment, 37.5 µl prewashed beads and 15 µg of HLA-I antibody (W6/32) (Abcam; catalog no.: 22432 or Novus Biologicals; catalog no.: NB100-64775) were added to the filtered lysates in each well of the 96-well plate and incubated end over end at 4 °C for 3 h. Following the incubation beads and lysates were transferred to a prewashed 10 µm PE fritted plate (Agilent; catalog no.: S7898A) stacked on top of a fresh 96 well, 2 ml deep plate. The flow-through from the HLA-I enrichment was snap-frozen and used for downstream proteome and PTMome analyses.

Beads containing either HLA-I or HLA-II peptide complexes were transferred and washed on a 10 µm PE fritted plate placed on a Positive Pressure-96 Processor (Waters; WT186006961). HLA peptides were acid eluted using 2x 3%ACN/5%FA and 2x 10% acetic acid (5 min incubation) directly onto an activated (500 µL methanol, 500 µL 99.9% ACN/0.1% FA) and equilibrated (4x 1000 µL 1% FA) tC18 40 mg Sep-Pak desalting plate (Waters; 186002320). 50 fmol of JPT iRT peptides were spiked into the samples during the acid elution steps. The HLA peptides were desalting using 4 x 1 mL 1% FA sand eluted from the Sep-Pak desalt plate using 250 µL 15% acetonitrile (ACN)/1% formic acid (FA) and 2× 250 µl of 50% ACN/1% FA for HLA-I and 60% ACN/1% FA for HLA-II into 96 deep-well plates, snap frozen, and lyophilized.

The dried HLA eluted peptides were stored at -80°C until reduction and alkylation were performed. HLA peptides were reconstituted in 100 µL 10mM Tris-HCl pH 7.5 (Invitrogen, Waltham, Massachusetts, USA, 15567027). 2 µL of 250 mM dithiothreitol (DTT) (Thermo Fisher Scientific, Waltham, Massachusetts, USA,

A39255) was added to the sample for a final concentration of 5 mM and incubated for 20 min at 50°C while shaking at 1000 rpm. 6 µL of 250 mM iodoacetamide (IAA)(Thermo Fisher Scientific, Waltham, Massachusetts, USA, A39271) was added to the sample for a final concentration of 15 mM and incubated for 30 min at room temperature while shaking at 1000 rpm in the dark. The reaction was quenched with 2 µL of 250 mM DTT, for a final concentration of 5 mM, and incubated for 15 min at room temperature while shaking at 1000 rpm. The sample was brought to a final concentration of 3% ACN/5% FA by adding 90 µL of 6.67% ACN/11% FA.

A secondary desalt was performed on the sample by mirco-scaled basic reversed phase separation on SDB-XC stage tips. Stage tips were set up with two punches of SDB-XC material (CDS analytical, previously Empore 3M, Oxford, Pennsylvania, USA, 98-0604-0223-1). Stage tips were equilibrated with 2x 100µL methanol, 100 µL 50% ACN/1% FA, and 3x 100 µL 1% FA. The lyophilized peptides were resuspended in 200 µL 3% ACN/5% FA, loaded onto the equilibrated stage tip, and ran through twice to maximize peptide retrieval. HLA-peptides were desalted with 3x 100 µL 1% FA. HLA-peptides were eluted from the SDB-XC stage tips with 60 µL 5% ACN/1% FA, 60 µL 10% ACN/1% FA, and 60 µL 50% ACN/1% FA for HLA-I and 60% ACN/1%FA for HLA-II. Combined elution fractions were frozen at -80°C, lyophilized, and stored at -80°C until LC-MS/MS analysis.

LC–MS/MS Analysis

Peptides were reconstituted in 3% ACN/5% FA prior to loading onto a 25 cm Aurora Ultimate CSI nanoflow analytical column with 1.7 µm particle size (IonOpticks, Fitzroy, Victoria, Australia). HLA peptides were loaded in solvent A (0.1% FA), separated with a linear and stepped gradient with the Bruker nanoElute ranging from 2 to 15 solvent B (0.1% FA in ACN) over 60 min, 15 to 23% in 30 min, 23 to 35% in 10 min, 35 to 80% in 10 min, and held at 80% for 10 min at 400 nl/min directly into an Bruker timsTOF SCP (SCP). MS1 scans were acquired from 100 to 1700 m/z and 1/K0 = 1.7 Vs cm−2 to 0.6 Vs cm−2 for HLA-I or 1/K0 = 1.3 Vs cm−2 to 0.6 Vs cm−2 for HLA-II in DDA-PASEF mode. Ten PASEF ramps were acquired with an accumulation and ramp time of 166 ms or as stated otherwise. Precursor above the minimum intensity threshold of 1000 were isolated with 2 Th at < 700 m/z or 3 Th >800 m/z and res-sequenced until a target intensity of 10,000 considering a dynamic exclusion of 40 s or as stated otherwise. The collision energy was lowered linearly as a function of increasing ion mobility from 55.00 eV at 1/K0 = 1.60 Vs/cm2 to 15.00 eV at 1/K0 = 0.60 Vs/cm2. Standard tryptic precursor isolation polygon placement was optimized for HLA-I peptide species by extending the polygon to include singly charged precursors with >600 m/z (PMID: 36993564).

HLA Peptide Search and Identification

Mass spectra were interpreted using the Spectrum Mill (SM) software package, version 8.01 (Broad Institute; proteomics.broadinstitute.org) as previously described (26, 32, 33) with modifications detailed later. Briefly, only MS/MS spectra with precursor sequence MH+ in the range 700 to 2000 for HLA-I and 700 to 4000 for HLA-II, a precursor charge 1 to 4 for HLA-I and 2 to 6 for HLA-II, or a minimum of <5 detected peaks were extracted. Merging of similar spectra with the same precursor m/z acquired in the same chromatographic peak was enabled. MS/MS spectra with a sequence tag length >1 (i.e., minimum of three masses separated by the in-chain masses of two amino acids) were searched with no-enzyme specificity; instrument: ESI-QEXACTIVEHCD-HLA-v3; fixed modifications: carbamidomethylation of cysteine; variable modifications: oxidation of methionine, pyroglutamic acid at peptide N-terminal glutamine, protein N-terminal acetylation and deamidation; precursor mass tolerance of ±10 ppm;

product mass tolerance of ±10 ppm for Exploris or ±15 ppm for SCP data; and minimum matched peak intensity (percent scored peak intensity or %SPI) of 40%. SPI is the percent of product ion intensity (after peak detection) that is matched to a scored ion type.

MS/MS spectra were searched against a compiled database comprised of the human reference proteome Gencode 42 (ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_34/42) with 47,429 or 50,872 nonredundant protein-coding transcript biotypes mapped to the human reference genome GRCh38, 602 common laboratory contaminants, 2043 curated small ORFs (lncRNA and upstream ORFs [uORFs]), 237,427 novel unannotated ORFs (nuORFs) supported by ribosomal profiling nuORF DB v1.037 for a total of 287,501 or 290,944 entries, respectively (PMID: 34663921).

Peptide spectrum matches (PSMs) within <1% false discovery rate (%FDR) using the target decoy estimation of the SM autovalidation module were filtered for a sequence length of 8 to 12 amino acids or 12 to 40, a minimum backbone cleavage score (BCS) of 5 or 7 for HLA-I or HLA-II peptides, respectively. BCS is a peptide sequence coverage metric to enforce a uniformly higher minimum sequence coverage for each PSM. The BCS is a sum after assigning a 1 or 0 between each pair of adjacent amino acids in the sequence (maximum score is peptide length −1) considering all selected ion types to decrease false-positive spectra having fragmentation in a limited portion of the peptide by multiple ion types.

PSMs were consolidated to peptides using the SM protein/peptide summary module case-sensitive peptide-distinct mode. A distinct peptide was the single highest scoring PSM of a peptide detected for each sample. Different modification states observed for a peptide were each reported when containing amino acids configured to allow variable modifications; a lowercase letter indicates the variable modification (C-cysteinylated and c-carbamidomethylated). In addition, precursor fragmentation was evaluated through the percent-dissociated intensity (PDI). The PDI reports the intensity of residual precursor and its neutral losses of water and ammonia subtracted from the total peak intensity in the MS/MS spectrum divided by the total peak intensity.

Identified peptides were filtered for common contaminants, peptides for which both the preceding and C-terminal amino acids were tryptic residues and peptides observed in negative control runs as described previously (PMID: 31844290, PMID: 28228285).

Subset-Specific FDR Filtering for nuORFs

While the aggregate FDR for was set to <1%, as described previously, FDR for subset of nuORFs (<5% of total of HLA-I peptides) required more stringent score thresholding to reach a suitable subset-specific FDR <1.0%. To this end, we devised and applied subset-specific filtering approaches. Subsets of nuORF types were thresholded independently in the HLA datasets using a two-step approach. First, PSM scoring metric thresholds were tightened in a fixed manner for all nuORF PSMs so that nuORF distributions for each metric improved to meet or exceed the aggregate distributions. For all ‘omes, the fixed thresholds were minimum score: 7, minimum %SPI: 50%, precursor mass error: ±5 ppm, minimum BCS: 5, and sequence length: 8 to 12 (HLA-I) and 9 to 50 (HLA-II). Second, individual nuORF type subsets with FDR estimates remaining above 1% were further subject to a grid search to determine the lowest values of BCS (sequence coverage metric) and score (fragment ion assignment metric) that improved FDR to <1% for each ORF type in the dataset.

HLA Peptide Prediction

HLA peptide prediction was performed using HLAthena (hlathena.tools). Unless otherwise specified, peptides were assigned to an allele using a percentile rank cutoff of ≤2.0.

References:

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2. Abelin JG, Bergstrom EJ, Rivera KD, Taylor HB, Klaeger S, Xu C, Verzani EK, Jackson White C, Woldemichael HB, Virshup M, Olive ME, Maynard M, Vartany SA, Allen JD, Phulphagar K, Harry Kane M, Rachimi S, Mani DR, Gillette MA, Satpathy S, Clauser KR, Udeshi ND, Carr SA. Workflow enabling deepscale immunopeptidome, proteome, ubiquitylome, phosphoproteome, and acetylome analyses of sample-limited tissues. Nat Commun. 2023 Apr 3;14(1):1851. doi: 10.1038/s41467-023-37547-0. PMID: 37012232; PMCID: PMC10070353.