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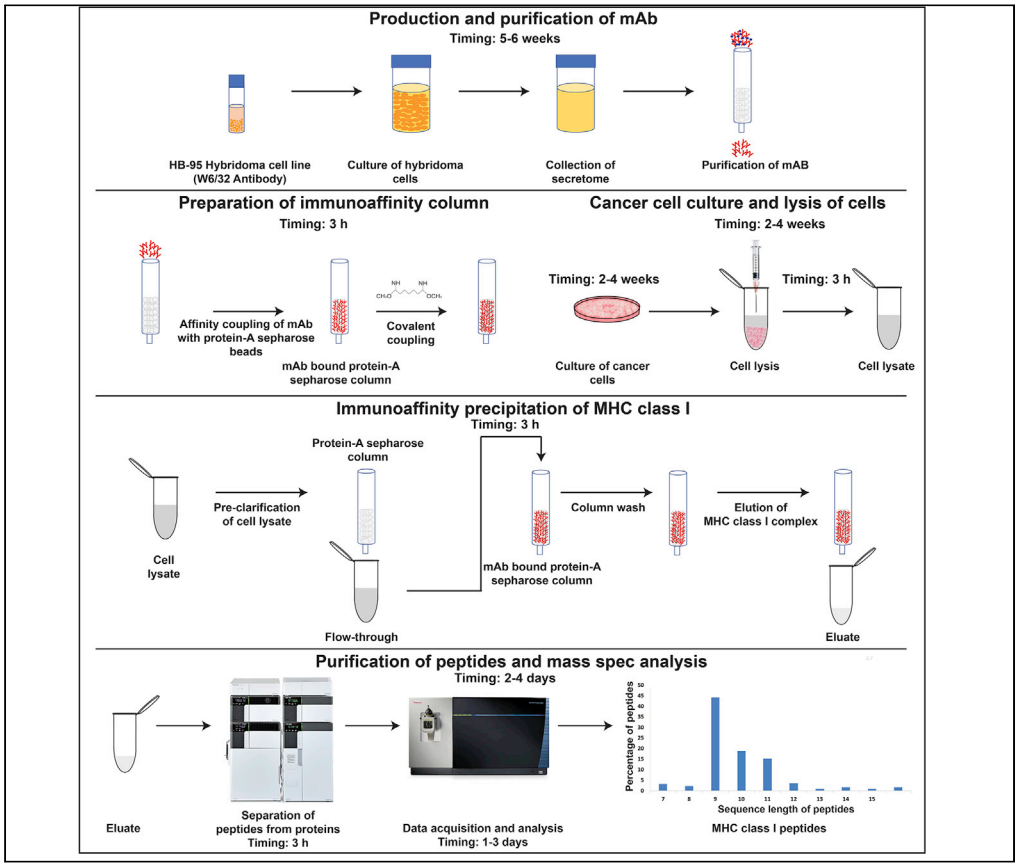
Protocol

Protocol for purification and identification of MHC class I immunopeptidome from cancer cell lines



Major histocompatibility complexes (MHC) play a critical role in immunity by presenting peptides on the cell surface for T cell recognition. Identification of these peptides can be valuable to develop vaccines or immunotherapeutic strategies for infectious diseases and cancers. Mass spectrometry is the only tool available for unbiased identification of the immunopeptidome. Here, we describe a protocol for purification and identification of MHC class I peptides, including in-house purification of anti-MHC-antibody from hybridoma cells and the LC-MS/MS analysis of MHC-I bound peptides.

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HIGHLIGHTS

In-house production and Sepharose conjugation of anti-MHC class I antibodies

Affinity purification of MHC-I complex from cultured cancer cell lysates

LC-MS/MS analysis of purified MHC-I bound peptides

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Protocol for purification and identification of MHC class I immunopeptidome from cancer cell lines

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SUMMARY

Major histocompatibility complexes (MHC) play a critical role in immunity by presenting peptides on the cell surface for T cell recognition. Identification of these peptides can be valuable to develop vaccines or immunotherapeutic strategies for infectious diseases and cancers. Mass spectrometry is the only tool available for unbiased identification of the immunopeptidome. Here, we describe a protocol for purification and identification of MHC class I peptides, including in-house purification of anti-MHC-antibody from hybridoma cells and the LC-MS/MS analysis of MHC-I bound peptides.

BEFORE YOU BEGIN

Note: Substantial amount (10–20 mg) of anti-MHC class I antibody is required to pull down MHC complex and identify bound peptides. This antibody is commercially available and can be purchased in bulk. However, if it is cost prohibitive, the following section provides step-by-step protocol to culture the hybridoma cells and purify the antibodies in-house

Hybridoma culture for antibody production

Timing: 5–6 weeks

1. Thaw one vial of W6/32 (HB-95) hybridoma cells in DMEM supplemented with 10% FBS at a density of 2e5/mL.
2. Change media on 2nd day and maintain them until they reach a density of 1e6/mL.
3. Split the cells at a ratio of 1:1 and change media from DMEM with 10% FBS to HyClone CDM4MAb serum free media (SFM).
4. Maintain cells until they reach a density of 1e6/mL.
5. Transfer the cells to Corning Roller Bottles and maintain them on constant agitation (1 revolution per minute) so that cells do not settle down.
6. Add 100 mL of SFM every day until the total volume reaches 1 L.
7. Keep the cells on rotation for 1 month.

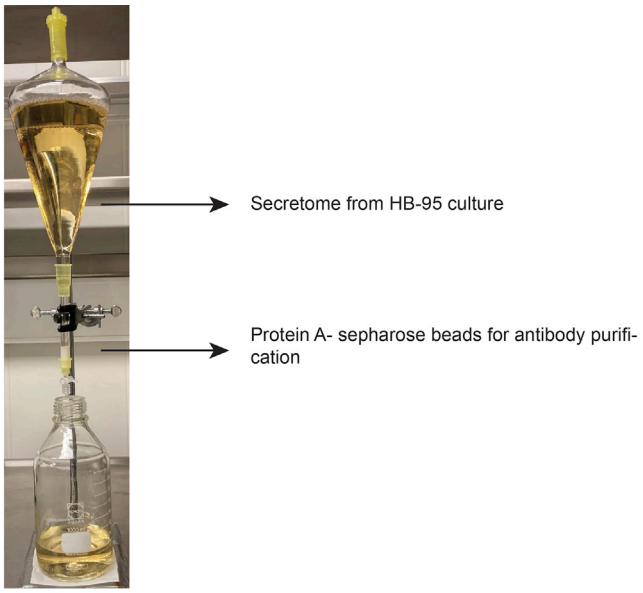

Figure 1. Setup for purification of MHC class I antibodies from secretome of HB-95 cells

8. After a month, pellet the cells down by centrifuging them at 300 × *g* for 10 min at 20°C.
9. Collect the secretome and filter using 0.2 μm Nalgene Rapid-Flow Sterile Single Use Vacuum Filter Units.

Purification of antibody W6/32 from cell secretome

Timing: 12–24 h

Note: Perform antibody purification at 4°C.

10. Transfer 4 mL of protein A Sepharose beads to a glass column.
11. Wash the column with 10 column volumes (c.v.) of PBS, pH 7.4.
12. Pass the secretome through the column (Figure 1).
13. Wash the antibody-bound protein A column with 50 c.v. of PBS.
14. Elute W6/32 antibody with 5 c.v. of 100 mM glycine/HCl, pH 3.0 and collect fractions in tubes containing 200 μL 2 M Tris-HCl, pH 7.5.
15. Wash the column with 10 c.v. of 50 mM Tris-HCl, pH 8.0.
16. Pass 20 c.v. of PBS through the column.

Note: The pH of solution flowing out of the column should be > 7.

17. Store the column in 0.02% sodium azide/PBS at 4°C.
18. Take 5 μL from each fraction and run an SDS-PAGE. Pool all fractions containing antibody (Figure 2).
19. Estimate the concentration and yield of antibody using NanoDrop or any protein estimation strategy.

⚠ CRITICAL: The final pH of antibody fractions collected should be between 7.5–8 to make sure antibodies are not denatured.

Note: The column prepared for purification can be re-used if stored at 4°C to avoid fungal growth. Antibodies can also be procured commercially

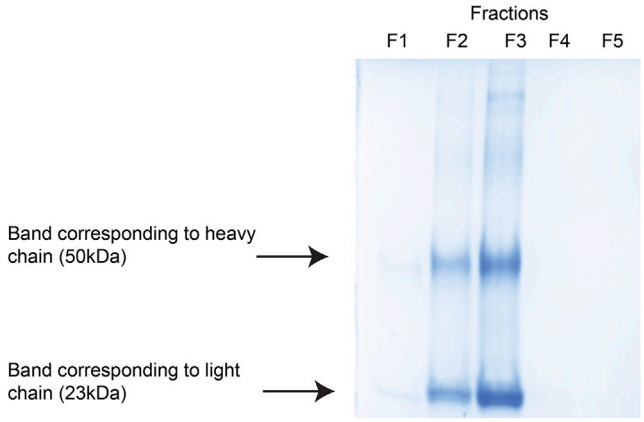

Figure 2. SDS-PAGE of fractions collected during antibody purification

Cancer cell line culture

Timing: 2–4 weeks

Note: Number of cells required to identify MHC bound peptides varies based on expression level of MHC, sensitivity of mass spectrometer, and antibody affinity during immunoprecipitation. Start the experiment with 250–500 million cells to achieve sufficient depth and coverage of MHC peptides.

20. Culture the cancer cell lines of interest in appropriate media
21. Harvest suspension cell lines as follows -
    a. Transfer the cells to centrifuge tubes.
    b. Take an aliquot of cell suspension to count the number of cells.
    c. Pellet the cells down by centrifuging them at 350 × *g* for 10 min at 20°C–25°C.
    d. Wash cell pellets 3 times with PBS at 20°C–25°C.
    e. Aspirate PBS and freeze the cell pellets at −80°C if you plan to carry out lysis and subsequent steps later.
22. Harvest the adherent cells using versene
    a. Aspirate cell culture media from the culture flask.
    b. Wash the cells with 2–5 mL versene solution and incubate the flask for 10 min at 20°C–25°C.
    c. Add PBS to the flask and harvest the cells.
    d. Take an aliquot of cell suspension to count the number of cells.
    e. Wash cell pellets 3 times with PBS at 20°C–25°C.
    f. Aspirate PBS and freeze the cell pellets at −80°C if you plan to carry out lysis and subsequent steps later.

⚠ CRITICAL: It is important to ensure cell viability of ≥90% after harvesting

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
InVivoMAb anti-human MHC Class I (HLA-A, HLA-B, HLA-C) (optional)	Assay Matrix Pty Ltd	Cat#BE0079
Chemicals, peptides, and recombinant proteins		
Tris hydrochloride	Sigma-Aldrich	Cat#10812846001
Hydrochloric acid 37% (HCl)	Sigma-Aldrich	Cat#320331-500ML

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium azide	Sigma-Aldrich	Cat#S2002-5G
Versene solution	Life Technologies	Cat#15040066
Boric acid	Sigma-Aldrich	Cat#B0394-100G
Potassium chloride (KCl)	Sigma-Aldrich	Cat#P9333-500G
Triethanolamine	Sigma-Aldrich	Cat#90279-100ML
Ethanolamine (optional)	Sigma-Aldrich	Cat# E9508-100ML
Sodium hydroxide (NaOH)	Sigma-Aldrich	Cat#221465-500G
Citric acid	Sigma-Aldrich	Cat#251275-100G
IGEPAL CA-630 (NP-40)	Sigma-Aldrich	Cat#I8896-50ML
Sodium deoxycholate	Sigma-Aldrich	Cat#D6750-10G
Sodium chloride	Sigma-Aldrich	Cat#S7653-250G
cOmplete Protease Inhibitor Cocktail	Merck	Cat#11697498001
Iodoacetamide (IAA)	Sigma-Aldrich	Cat#I1149-5G
CaptivA Protein A Affinity Resin	REPLIGEN	Cat#CA-PRI-0025
Dimethyl pimelimidate dihydrochloride (DMP)	Sigma-Aldrich	Cat#D8388-1G
LM- CDM4MAb W/ GLUT	GE Healthcare Life Sciences	Cat#SH30801.01
DMEM	Life Technologies	Cat#11995073
RPMI	Life Technologies	Cat#11875119
FBS	Life Technologies	Cat#10437-028
Acetonitrile, Optima LC/MS grade, Fisher Chemical	Fisher Scientific	Cat# A955-212
Water, Optima LC/MS grade, Fisher Chemical	Fisher Scientific	Cat# W6212
Trifluoroacetic acid, Optima LC/MS grade, Fisher Chemical	Fisher Scientific	Cat# A116-50
Formic acid, 99.0+%, Optima LC/MS grade, Fisher Chemical	Fisher Scientific	Cat# A117-50
Experimental models: cell lines		
ATCC W6/32; hybridoma; mouse (*Mus musculus*) BSL1, 1 mL	ATCC	ATCHB95
Software and algorithms		
Table S1	This study	Mendeley data (https://data.mendeley.com 10.17632/4bk7w8298s.1)
Mass spectrometry raw file	This study	Mendeley data (https://data.mendeley.com 10.17632/4bk7w8298s.1)
Proteome Discoverer Suite (2.2)	Thermo Scientific	https://www.thermofisher.com/store/products/OPTON-30945#/OPTON-30945
Mascot	Matrix Science	https://www.matrixscience.com/
Peaks X+	N/A	https://www.bioinfor.com/peaks-studio/
MaxQuant	Cox and Mann, 2008	https://www.maxquant.org/
Seq2Logo 2.0	Thomson and Nielsen, 2012	http://www.cbs.dtu.dk/biotools/Seq2Logo/
NetMHCpan 4.1	Reynisson et al., 2020	http://www.cbs.dtu.dk/services/NetMHCpan/
SYEPEITHI	Schuler et al., 2007	http://www.syfpeithi.de/
Other		
WaterXBridge column	Waters	Cat# 186008982
Acquity UPLC M-Class V/M symmetry trap column	Waters	Cat# 186007496
Acquity UPLC M-Class Peptide BEH C18 nanoAcquity Column 10K psi	Waters	Cat# 186003544
SpeedVac vacuum concentrator		
Orbitrap Fusion Lumos Tribrid Mass Spectrometer	Thermo Scientific	NA
LC-MS certified total recovery vial	Waters	Cat# 600000671CV
HPLC with fraction collector	NA	NA
HPLC vials	Shimadzu	Cat# 226-50512-00
HPLC vial inserts	Shimadzu	Cat# 226-50523-00

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Waters Acquity UHPLC	Waters	NA
Needles 18G × 38 mm	NA	NA
10–50 mL syringes	NA	NA
ECONO column 1 × 10, 2/Pack	Bio-Rad	Cat#7371012
Protein LoBind tubes	Eppendorf	Cat#0030108116
0.2 μm Nalgene rapid-flow sterile single use vacuum filter units	Thermo Fisher Scientific	Cat#153-0020
Millex-HV syringe filter unit, 0.45 μm, PVDF, 33 mm	Merck	Cat# SLHV033RS
Corning 50 mL centrifuge tubes	Merck	Cat# CLS4558-300EA
Corning roller bottles, CellBIND cell culture surface, bottle surface area 1,700 cm^2	Merck	Cat# CLS431134
cRAP protein sequences		https://www.thegpm.org/crap/
HLA allele expressed in cell lines	ATCC	https://www.atcc.org/
	Cellosaurus	https://web.expasy.org/cellosaurus/
NanoDrop	NA	NA
Refrigerated centrifuge capable of achieving 20,000 ×*g*	NA	NA

MATERIALS AND EQUIPMENT

Borate wash buffer: 0.5 M boric acid and 0.5 M KCl, adjust the pH to 9 with NaOH. Add 0.31 g of boric acid and 0.38 g of KCl in 50 mL of water. Adjust pH to 9 with NaOH.

0.2 M Ethanolamine, pH 8 – Add 2.7 mL of triethanolamine to 100 mL of Milli-Q water and 1 mL of 33% HCl. Check the pH. If pH >8.3 add more HCl and if it is less than 8 adjust the pH with 10% NaOH

Note: pH range of 7.9–8.3 is acceptable to proceed further

40 mM DMP/0.2 M Ethanolamine, pH 8.3: Add 100 mg of DMP in 9 mL of 0.2 M ethanolamine. The resulting pH will be around 7. Add 200 μL of 50% NaOH and check the pH. If pH is <8.2 adjust it with NaOH and if it is >8.4 adjust the pH with HCl

Note: The rate of reaction is highest at pH 8.3. 5 mL of this solution is sufficient to bind 10 mg of antibody to 1 mL of protein A Sepharose beads.

⚠ CRITICAL: NP-40, sodium deoxycholate, IAA, TFA, formic acid, and acetonitrile are harmful and toxic. Work with these reagents in a chemical hood. Ethanolamine can be used as a substitute for triethanolamine. If working with ethanolamine, add 1.2 mL of ethanolamine to 60 mL of Milli-Q water. Add 1 mL of 33% HCl and check the pH. If pH is <8.2 adjust it with NaOH and if it is >8.4 adjust the pH with HCl

STEP-BY-STEP METHOD DETAILS

Generation of MHC immunoaffinity column

Timing: 3 h

The following steps describe the preparation of an immunoaffinity column for MHC class I pull-down.

Note: Wash the glass column with 1% TFA before packing the column for the first time to ensure no contamination from the glass column comes into the antibody-Sepharose column or in pre-column packed with Sepharose beads only.

1. Transfer the required amount of protein A Sepharose to a 15 mL Falcon tube and wash the beads three times with 10 mL PBS.

   Note: Conjugate 10–20 mg of W6/32 antibody per mL of protein A Sepharose beads.

2. Dilute the antibody solution to a concentration of 0.5–2 mg/mL in PBS.
   a. Retain an aliquot of 5 μL of antibody solution and label it as pre-mAb.
3. Add antibody to protein A Sepharose beads. Rotate gently end-over-end at 20°C–25°C for 1 h to allow binding.
4. Transfer the antibody-bound Sepharose beads to a glass column.
5. Collect the flow through in a fresh tube.
   a. Retain 5 μL of this flow through and label as post-mAb.
6. Wash antibody-bound Sepharose beads with 10 c.v. of borate wash buffer at 20°C–25°C.
7. Wash antibody-bound Sepharose beads with 10 c.v. of 0.2 M triethanolamine, pH 8.2, at 20°C–25°C.
8. Add 4–5 c.v. of freshly prepared 40 mM DMP cross-linker to the column and let it pass through until only a small meniscus is left at the top of the Sepharose beads. Allow the reaction to proceed at 20°C–25°C for 1 h.
9. Wash the antibody-bound Sepharose beads with 10 c.v. of ice-cold 0.2 M Tris-HCl, pH 8.0, at 20°C–25°C.
10. Wash the antibody-bound Sepharose beads with 10 c.v. of 0.1 M citrate buffer, pH 3. at 20°C–25°C.
11. Wash the antibody-bound Sepharose beads with 10 c.v. of PBS, at 20°C–25°C.
12. Run an SDS-PAGE for the samples aliquoted at steps 2 and 5. Both the heavy chain and light chain bands should be present in the pre-mAb sample and absent in post-mAb.

Troubleshooting 1

⚠ CRITICAL: Maintaining the pH of triethanolamine at 8.2 is important.

Pause point: The antibody column can be stored at 4°C for 2–3 months in the presence of 0.02% sodium azide/PBS

Preparation of Sepharose protein A column for pre-clearing of cell lysate

Timing: 3 h

This section describes the steps to make a column for pre-clearing the lysate before IP.

13. Transfer 1 mL protein A Sepharose beads to glass column.
14. Wash the column with 10 c.v. of PBS.

Note: The pre-column can be used 10 times or till the column gets blocked.

Pause point: This column can be stored at 4°C for 2–3 months in the presence of 0.02% sodium azide/PBS.

Preparation of cell lysate

Timing: 3 h

This section describes steps involved in preparation of cell lysate.

Table 1. Composition of cell lysis buffer

Cell lysis buffer (50 mL)	Final concentration	Amount
500 mM Tris-HCl buffer, pH 8	50 mM	5 mL
NP-40	1%	500 µL
Sodium deoxycholate	0.25%	125 mg
NaCl	150 mM	0.88 mg
IAA	20 mM	185 mg
cOmplete Protease Inhibitor Cocktail	-	1 tablet
Total	n/a	50 mL

15. Add 2 mL cell lysis buffer (Table 1) for 100 M cells and let it thaw on ice.
16. Homogenize the sample using 18G × 38mm needle. Repeat this 4–5 times in 15 min intervals.
17. Centrifuge the lysate at 2,000 × *g* at 4°C for 10 min.
18. Transfer the supernatant to a fresh tube and centrifuge it at 20,000 × *g* for 60 min at 4°C.
19. Pass the supernatant obtained in step 18 through a 0.45 µm filter to remove any debris and aliquot 30 µg equivalent cell lysate in a microcentrifuge tube and label it as pre-IP.

Note: Lysis of cells should be performed at 4°C. Do not store the cell lysate for more than 12 h as proteins tend to aggregate. Best practice is to lyse the cells and immediately carry out immunoprecipitation

Optional: Combine the pellets obtained in steps 17 and 18. Add 2% SDS/50 mM TEABC, pH 8.0 to lyse the membrane bound organelles obtained. Sonicate and centrifuge it at 20,000 × *g* for 30 min at 4°C. Perform western blot on the lysate obtained after adding 2% SDS/50 mM TEABC and at step 19, using anti-MHC class I antibody. MHC class I band corresponding to 40 kDa should be in the lysate from step 19 while it should be absent in the cell lysate obtained with SDS.

Immunoprecipitation of MHC class-I complex

Timing: 2–3 h

This section describes steps for carrying out immunoprecipitation of MHC class I complex from cell lysate

Note: Same approach can be used for carrying out immunoprecipitation of MHC class I complex from tissue lysate

20. Pass 10 c.v. of PBS through protein A Sepharose pre-column to remove sodium azide.
21. Pass cell lysate obtained in step 19 through the pre-column.
22. Collect flow through in a fresh tube.
23. Pass 10 c.v. of PBS through the antibody column to remove sodium azide.
24. Pass 5 c.v. of cell lysis buffer (without detergent) through the antibody column.
25. Pass the pre-cleared cell lysate obtained in step 22 through the antibody column and collect flow through in a fresh tube.
26. Pass flow through obtained in step 25 through the antibody column.
    a. Collect flow through in a fresh tube.
    b. Aliquot 30 µg of flow through to a microcentrifuge tube and label it as post-IP.
27. Wash antibody column with 10 c.v. of cell lysis buffer (without detergent).
28. Wash antibody column with 10 c.v. of 50 mM Tris, pH 8.0, and 150 mM NaCl.
29. Wash antibody column with 10 c.v. of 50 mM Tris, pH 8.0, 450 mM NaCl.
30. Wash antibody column with 10 c.v. of 50 mM Tris, pH 8.0, and 150 mM NaCl.
31. Wash antibody column with 10 c.v. of 50 mM Tris, pH 8.0.

Table 2. Solvent A and B for separation of peptides from MHC-I complex (for step 34)

HPLC solvents	LC-MS grade water (mL)	LC-MS grade ACN (mL)	TFA (mL)	Total (L)
Solvent A	999	0	1	1
Solvent B	199	800	1	1

32. Elute the proteins bound on antibody column with 10% (v/v) acetic acid in 10 fractions of 500 μL each.

Note: Regenerate the column by passing 10 c.v. of 100 mM Tris-HCl, pH 8.0 and then 10 c.v. of PBS. The pH of liquid flowing out from the column should be more than 7.

33. Pool all fractions collected in step 32.

Troubleshooting 2

Pause point: The samples can be stored for a couple of days without much loss.

Note: Clear the protein from pre-column by washing the column with 5 c.v. of 10% acetic acid and regenerate by passing 10 c.v. of 100 mM Tris-HCl, pH 8.0 followed by 10 c.v. of PBS. The pH of liquid flowing out from the column should be more than 7. If reusing pre-column, ensure no protein is left on the column by running later fractions of acid wash on SDS-PAGE. Flow through can be run on SDS-PAGE to ensure no protein is bound to pre-column to avoid cross contamination.

Optional: Perform western blot for the samples labeled pre- and post-IP using anti-HLA-A/B/C antibody. 40 kDa band should be detectable in pre-IP sample and absent in post-IP sample

Purification of peptides from MHC- class I complex by reverse phase liquid chromatography

Timing: 1–3 h

This section describes the steps and parameters for purifying peptides from MHC complex.

34. Set the flow rate of HPLC system to 2 mL/min (Table 2).
35. Equilibrate the column with 2% Solvent B.
36. Add trifluoroacetic acid (TFA) to the sample obtained at step 33 such that the final concentration of TFA is 1%.
37. Transfer the eluate to auto sampler vial.
38. Resolve peptides using the following LC gradient:

Time (min)	Flow (mL/min)	% A	% B
0.01	2	98	2
0.5	2	85	15
4.5	2	70	30
12.5	2	60	40
22.5	2	55	45
24.5	2	0	100
26.5	2	0	100
27	2	98	2
33	2	98	2

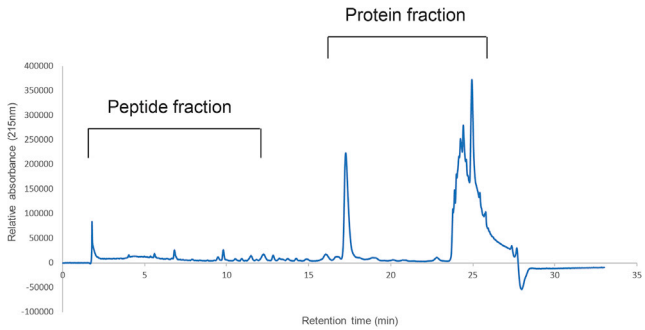

Figure 3. Separation of peptides from proteins using high performance liquid chromatography

39. Collect 500 μL fractions in micro centrifuge tubes.
40. Concatenate the fractions into 6 samples by combining fractions eluted at different hydrophobicity range (Figure 3).
41. Concentrate the samples using speedvac.

Note: Use protein low bind tubes to concentrate samples. Do not dry the samples completely. Concentrate the samples to 5–10 μL

Troubleshooting 3

Pause point: Samples can be stored after this step at −80°C

Mass spectrometry analysis of peptides eluted from MHC complex

Timing: 1 day

This section describes the parameters to be used to acquire data on a Thermo Scientific Orbitrap Fusion mass spectrometer paired with a Waters nanoAcquity UHPLC.

42. Centrifuge the samples at 15,000 × *g* for 15 min.
43. Transfer the samples to total recovery glass vials or 96-well plate compatible with the LC connected to mass spectrometer.
44. Set nanoLC flow rate to 300 nL/min (Table 3).
45. Load the peptides on trap column at 5 μL /min using 5% solvent B.
46. Resolve peptides on analytical column using the following gradient:

Time (min)	Flow (μL /min)	% A	% B
Initial	0.300	92	8
44	0.300	70	30
49	0.300	30	70
54	0.300	30	70
55	0.300	95	5
60	0.300	95	5

47. Acquire mass spectrometry data using following parameters:
    a. MS1: Resolution – 60,000; Mass Range – 350–1,800 *m/z*; AGC Target – $1e^6$, Maximum injection time 50 ms.

Table 3. Solvent A and B for nano-flow LC for mass spec analysis (for step 44)

LC-MS solvents	LC-MS grade water (mL)	LC-MS grade ACN (mL)	Formic acid (mL)	Total (mL)
Solvent A	200	0	0.2	200
Solvent B	0	200	0.2	200

b. Precursor selection: Top-speed mode with 2 s cycle time (for top N mode, set N = 10), Include charge state – 1–4; Dynamic exclusion – 5 s
c. MS2: Isolation window – 1.2 *m/z*; Activation type – HCD, Collision energy mode – Stepped; HCD Collision Energy – 25,27,30; Orbitrap resolution – 15,000; AGC target – $2e^5$, Maximum injection time – 120 ms

Note: Data acquisition parameters vary based on the instrument on which data is acquired. The above parameters are for Orbitrap Fusion mass spectrometer

Database searches of mass spectrometry data

Timing: 3 h to 2 days

This section describes mass spectrometry data analysis using Proteome Discoverer suite 2.2 with Sequest/Mascot as search engine

48. Select spectra based on following parameters: Lowest charge state – 1; Highest charge state – 4; Minimum precursor mass – 350 Da; maximum precursor mass – 5,000 Da

49. Sequest/Mascot search parameters:
    a. Database: Reference protein database of relevant species.

    Note: Add cRAP protein sequences (common Repository of Adventitious Proteins) that are commonly found as contaminant proteins in a proteomics experiment
    b. Enzyme Name: No-Enzyme (Unspecific) on SequestHT, NoCleave on Mascot
    c. Precursor Mass tolerance: 10 ppm
    d. Fragment mass tolerance: 0.05 Da
    e. Dynamic modifications: Oxidation on Methionine, Carbamidomethylation on C

50. 1% FDR at PSM and Peptide level

Note: For identifying MHC peptides with post translational modifications like phosphorylation at S/T/Y, deamidation at N/Q/R can be given as variable modifications for MS/MS search.

Troubleshooting 4

Troubleshooting 5

Optional: Other MS/MS search algorithms like Peaks and MaxQuant (Cox and Mann, 2008) can also be used. If carrying out mutant/non-canonical peptide analysis, include relevant protein sequences along with reference protein database in step 49a.

Data analysis and interpretation

Timing: 0.5–24 h

This step describes downstream analysis of MHC peptides

51. Remove peptides originating from known contaminant proteins to get the list of MHC presented peptides.
52. Carry out sequence length analysis of peptides. Majority of peptides should be between 8 to 14 amino acids.
53. Motif analysis can be done using tools such as Seq2Logo 2.0 (Thomson and Nielsen, 2012), SYEPEITHI (Schuler et al., 2007)
54. NetMHCpan-4.1 (Reynisson et al., 2020) can be used to predict the binding affinity of peptides to MHC class I molecule.

Note: For many cancer cell lines, HLA allele information can be found at Cellosaurus, ATCC website, or HLA typing can be performed for the cell lines.

EXPECTED OUTCOMES

Thousands of peptides can be identified by following the above protocol (number of peptides identified depends on expression level of MHC complex, efficiency of MHC pull-down, and sensitivity of mass spectrometer). Using the protocol, we identified 1,300 peptides from 25 million SK-MEL-28 cells (Table S1). Using more cells as starting material will result in identification of more MHC bound peptides.

LIMITATIONS

This protocol is applicable for identifying MHC class I peptides for cell line based system and can be expanded for MHC class II peptides by selecting an appropriate antibody for carrying out immunoprecipitation of MHC class II. As MHC class II peptides are longer, data acquisition parameters need to be modified accordingly. The protocol can also be adapted for immunopeptidome studies using primary cells or tissues. However, some optimization may be required to overcome limitations of cell numbers or limited tissue availability

TROUBLESHOOTING

Problem 1

Covalent cross linking of antibody to the Sepharose beads failed (see "Generation of MHC immunoaffinity column").

Potential solution

Check the pH of 0.2 M triethanolamine. If the pH of triethanolamine is >10, it could result in stripping of antibody before the coupling reaction. Also, pH 8.3 of DMP-triethanolamine is critical for the coupling reaction. The efficiency of the reaction reduces with decrease or increase of pH.

Problem 2

HLA band is detectable in post IP sample (see "Immunoprecipitation of MHC class-I complex").

Potential solution

Make a new column with fresh antibody and Sepharose beads.

Problem 3

The resolution of separation of peptides from proteins during offline fractionation was poor (see "Purification of peptides from MHC- class I complex by reverse phase liquid chromatography").

Potential solution

The bRPLC method given here works best for the Waters XBridge column. If using a different column, the gradient method may have to be optimized again. Also, TFA gives better resolution

than FA. It is advisable to use 0.1% TFA than 0.1% FA to acidify the solvents. Equilibrate the column with the same gradient before injecting the sample of interest.

Problem 4

Identification of many tryptic peptides outside the expected size range for MHC bound peptides (see "Database searches of mass spectrometry data").

Potential solution

It could be carryover from previous mass spectrometry runs on the same column. Flush the column with high organic to remove peptides bound to column, equilibrate, and then load MHC peptidome for mass spectrometry analysis

Problem 5

Number of MHC peptides identified was considerably less than expected (see "Database searches of mass spectrometry data").

Potential solution

1. Check the expression level of MHC class I complex in the sample of interest. If MHC expression level is low, increase the number of cells.
2. Check the efficiency of IP by running a western blot of pre and post IP samples.
3. Check whether the mass spec is running at optimal condition by acquiring a quality control sample.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Harsha Gowda (harsha.gowda@qimrberghofer.edu.au).

Materials availability

This study did not generate new unique reagents

Data and code availability

Raw and processed mass spectrometry data presented in this protocol is available on Mendeley data (https://data.mendeley.com) with https://doi.org/10.17632/4bk7w8298s.1.

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AUTHOR CONTRIBUTIONS

S.V.M. optimized the protocol and conducted experiments. S.V.M. and R.Z. carried out fractionation and mass spectrometry data acquisition. S.V.M. and K.K.D. carried out data analysis. K.K.D., C.S., and H.G. supervised the study. S.V.M. wrote the first draft. K.K.D., C.S., and H.G. critically read and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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