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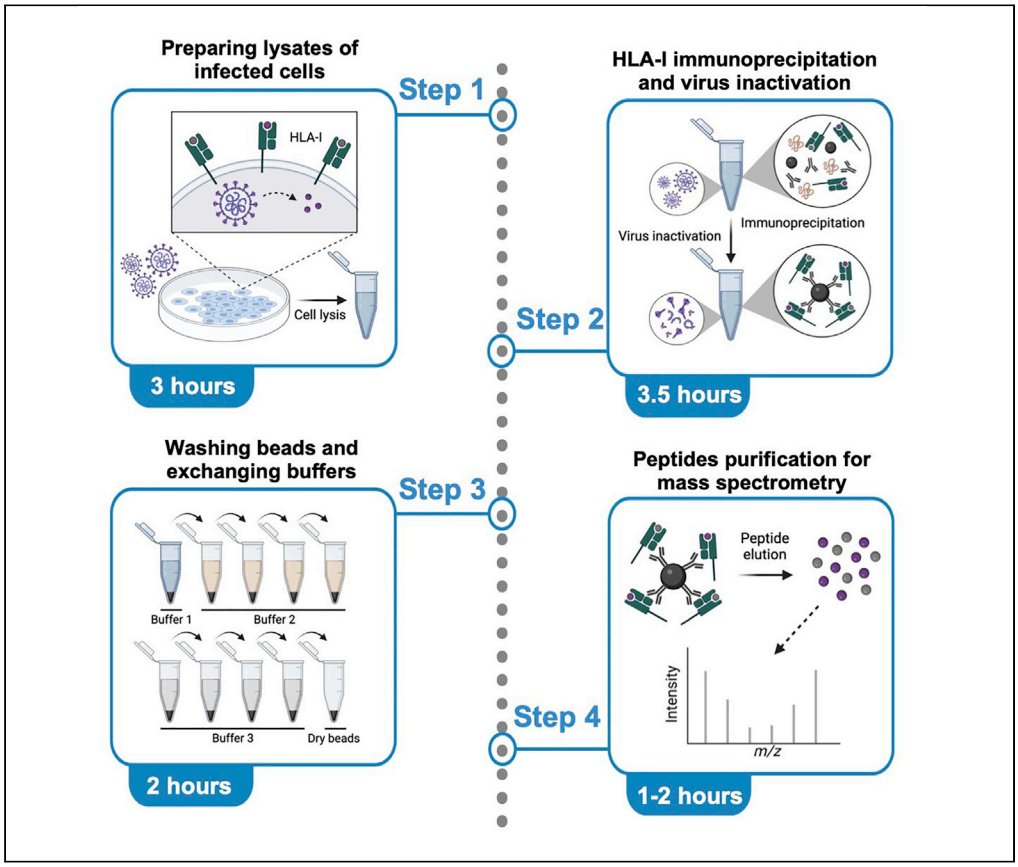
HLA-I immunopeptidome profiling of human cells infected with high-containment enveloped viruses



Immunopeptidome profiling of infected cells is a powerful technique for detecting viral peptides that are naturally processed and loaded onto class I human leukocyte antigens (HLAs-I). Here, we provide a protocol for preparing samples for immunopeptidome profiling that can inactivate enveloped viruses while still preserving the integrity of the HLA-I complex. We detail steps for lysate preparation of infected cells followed by HLA-I immunoprecipitation and virus inactivation. We further describe peptide purification for mass spectrometry outside a high-containment facility.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Profiling naturally presented HLA-I peptides in infected cells by mass spectrometry

Immunoprecipitating HLA-I complexes from cells and purifying bound peptides

Inactivating enveloped viruses while preserving the integrity of the HLA-I complex

Detecting HLA-I peptides that are derived from host and viral proteins

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HLA-I immunopeptidome profiling of human cells infected with high-containment enveloped viruses

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SUMMARY

Immunopeptidome profiling of infected cells is a powerful technique for detecting viral peptides that are naturally processed and loaded onto class I human leukocyte antigens (HLAs-I). Here, we provide a protocol for preparing samples for immunopeptidome profiling that can inactivate enveloped viruses while still preserving the integrity of the HLA-I complex. We detail steps for lysate preparation of infected cells followed by HLA-I immunoprecipitation and virus inactivation. We further describe peptide purification for mass spectrometry outside a high-containment facility.

For complete details on the use and execution of this protocol, please refer to Weingarten-Gabbay et al. (2021).[1]

BEFORE YOU BEGIN

This protocol describes in detail the methods and resources needed for mass spectrometry analysis of HLA-I peptides in cells infected with a high containment enveloped virus. Traditional inactivation methods, such as Trizol, SDS and heating, destroy the structural conformation of the HLA-I complex and are therefore incompatible with immunopeptidome profiling. In contrast, mild detergents can inactivate an enveloped virus by disrupting its membrane while still maintaining the integrity of the HLA-peptide complex in the lysates. In the protocol below, virus inactivation occurs during the HLA-I immunoprecipitation step, when infected cells are incubated with a lysis buffer containing Triton-X detergent.

This protocol was modified from Abelin et al.[2], Keskin et al.[3], and Reinhold et al.[4], and describes all experimental steps from virus infection through purified HLA peptides ready for mass spectrometry analysis. While this protocol was designed with high containment viruses in mind (viruses studied in a Biosafety Level 3 (BSL3) laboratory), it can also be used to profile HLA-I peptides of viruses that require less stringent containment conditions (viruses studied in a BSL2 laboratory) as well as non-infected cells.

The protocol described here centers around our work with human lung A549 cells and human embryonic kidney HEK293T cells infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). However, we also used it with HEK293T cells infected with Vesicular Stomatitis Virus (VSV). When applying it to a new enveloped virus, viral inactivation should be confirmed using appropriate assays and safety standards. Lysis buffer volume and incubation time required for inactivation may vary between viruses.

Preparing buffers

Timing: 2.5 h

Recipes for all buffers listed below can be found in the materials and equipment section.

1. Prepare Lysis Buffer Base.
2. Prepare Octyl β-d-glucopyranoside Stock Solution.
3. Prepare 500 mM Iodoacetamide aliquots.
4. Prepare Lysis Wash Buffer without Protease Inhibitor (-PI).
5. Prepare Lysis Buffer with Protease Inhibitor (+PI).
6. Prepare Wash Buffer Base.
7. Prepare Complete Wash Buffer.
8. Prepare 20 mM Tris Wash Buffer.

Note: Wash Buffer Base, Complete Wash Buffer and 20 mM Tris Wash Buffer (buffers 6–8) can either be made at the outset of the protocol alongside the other buffers, or during the 3 h incubation in the immunoprecipitation step (step 4 of the protocol).

Setting a rotisserie-style rotator inside a 4°C mini fridge in the BSL3 facility

Timing: 15 min

9. Place a tube rotator and rotisserie (see materials and equipment) inside a 4°C mini fridge in the BSL3 facility (Figure 1). Turn it on for 10 min before loading tubes with samples in step 4 of the protocol, set speed to 15 rpm.

Infecting cells with a virus

Timing: 2 days (48 h prior HLA-I immunoprecipitation)

10. Seed A549 cells in three 15 cm dishes at a density of 1.5 × 10^7 cells per dish to achieve ~80% confluency (2 × 10^7 cells per dish for HEK293T) 24 h prior to infection.
11. On the day of infection, carry cells to the BSL3 space and infect with SARS-CoV-2.
    a. For infection, remove the culture media and rinse the cell monolayer with 10 mL of ice-cold 1× PBS.
    b. Prepare virus dilution in 5 mL of ice-cold Opti-MEM to obtain the final multiplicity of infection (MOI) of 3, add to the cells, and allow viral adsorption on ice for 60 min.

Figure 1. Images of a tube rotator and rotisseries inside a 4°C mini fridge
Rotator was installed at the bottom shelf and the electric cord was connected to a socket outside the mini fridge.

c. During on-ice incubation, rock the dishes gently every 10 min to facilitate homogenous infection and prevent parts of the cell monolayer from drying.
d. After 1 h, remove the virus inoculum and add 25 mL of pre-warmed DMEM containing 2% FBS.
e. Incubate at 37°C in the presence of 5% CO2.

Note: Aim for high MOI to increase the relative representation of viral peptides in comparison to endogenous human HLA-I peptides.

Note: HLA-I presentation dynamics vary between viruses and cell lines. Some studies profiled the HLA-I peptidome late during infection, such as 2 days post infection (dpi) for measles virus,[5] 2–4 dpi for human immunodeficiency virus,[6] and 0.5–5 dpi for West Nile virus.[7] We and others[1,8,9] have shown that some viral HLA-I peptides peak as early as 3–6 h post infection (hpi). For SARS-CoV-2, most of the peptides we detected at 3–6 hpi were also present at 24 hpi.

Pre-washing beads before entering the BSL3 facility

Timing: 30 min (on the day of HLA-I immunoprecipitation)

12. Prepare GE Healthcare Gamma-bind plus Sepharose beads for immunoprecipitation (IP). The steps below describe the amount needed for 6 IPs, which constitute a single experiment.
    a. Fill 6 microcentrifuge tubes to the rim with 1× PBS to "soak" tubes.
    b. Pipette 1 mL of 1× PBS into a new 1.5 mL microcentrifuge tube and add 180 μL of beads (30 μL of beads per IP reaction).
    c. Vortex the tube for 7 s to wash the beads followed by centrifugation at 2,500 rcf for 30 s at 4°C in a pre-chilled swing bucket centrifuge. Aspirate the supernatant without disturbing the bead pellet. Repeat this step 2 times.
    d. After washing for three times, completely aspirate the supernatant (preferably by using a vacuum aspirator). Add 1 mL of 1× PBS and vortex to mix.
    e. Remove PBS from 6 "soaked" microcentrifuge tubes (from step 12a) and add 1 mL of fresh 1× PBS to each tube.
    f. Add 166 μL of washed beads to each of the 6 tubes obtaining the total volume of 1,166 μL per tube. Clearly label each tube with the date and sample name. At the end of the protocol, these tubes will be stored at −80°C before submission for mass spectrometry analysis.

Note: When collecting beads, cut the tip of the pipette with a disposable scalpel and rinse it 6 times with 1× PBS before loading beads.

Note: Use low retention tubes and keep beads on ice.

Note: When washing beads, use a swing bucket centrifuge. Fixed-angle rotor results in beads' smear on the side of the tube.

Preparing reagents and consumables to take to the BSL3 facility

Timing: 15 min (on the day of HLA-I immunoprecipitation)

13. Prepare an ice bucket with the following reagents and tubes. The steps below describe the amount needed for 6 IPs, which constitute a single experiment.
    a. Ice-cold Lysis Buffer (+PI) (after adding Phenylmethanesulfonylfluoride (PMSF)) - 8 mL in a 15 mL conical tube.
    b. 3 cell scrapers.
    c. 6 pre-soaked microcentrifuge tubes (filled to the rim with 1× PBS) for cell lysates.
    d. 6 microcentrifuge tubes containing pre-washed beads (from step 12).
    e. 10 µL Benzonase (1 µL per IP reaction plus extra).
    f. 350 µL HLA Class I Antibody W6/32 (50 µL per reaction plus extra).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HLA Class I Antibody W6/32 (200 µg/mL)	Santa Cruz Biotechnology	Cat # sc32235
FITC anti-human HLA-A,B,C Antibody (W6/32 clone)	BioLegend	Cat # 311403
Bacterial and virus strains		
SARS-CoV-2	Centers for Disease Control and Prevention and BEI Resources	2019-nCov/USA-WA1/2020 isolate (NCBI accession number: MN985325.1)
Chemicals, peptides, and recombinant proteins		
1 M Tris, pH 8.0	Invitrogen	Cat # AM9855G
1 M Magnesium Chloride	Sigma-Aldrich	Cat # M1028
0.5 M EDTA	Sigma-Aldrich	Cat # 7789
5 M Sodium Chloride	Sigma-Aldrich	Cat # 71376
Triton-X	Sigma-Aldrich	Cat # T9284
Octyl β-d-glucopyranoside	Sigma-Aldrich	Cat # 08001-5G
Phenylmethanesulfonylfluoride (PMSF)	Sigma-Aldrich	Cat # 78830
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	Cat # 11873580001
Gammabind Plus Sepharose Beads	Millipore Sigma	Cat # 17-0886-01
Iodoacetamide	Sigma-Aldrich	Cat # A3221
Benzonase	Thomas Scientific	Cat # E1014-25KU
Opti-MEM Reduced Serum Medium	Fisher Scientific	Cat# 31985-070
DMEM, high glucose, pyruvate	Fisher Scientific	Cat# 11995-065
PBS, pH 7.4	Fisher Scientific	Cat# 10010-023
Internal retention time (iRT) standards	Biognosys	SKU: Ki-3002-2
Experimental models: Cell lines		
A549	ATCC	CCL-185
HEK293T	ATCC	CRL-3216
VERO E6	ATCC	CRL-1586

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
DNA LoBind 1.5 mL Tube	Eppendorf	Cat# 022431021
Gel Loading Pipet Tips, 0.5 µL–10 µL	Fisher Scientific	Cat# 02-707-88
Falcon 150 mm TC-treated Cell Culture Dish	Corning	Cat# 353025
Corning Cell Lifter	Corning	Cat# 3008
Sep-Pak Vac 1cc (50 mg) tC18 Cartridges	Waters	SKU: WAT054960

MATERIALS AND EQUIPMENT

Equipment

During the immunoprecipitation step, lysates are incubated with beads and antibodies for 3 h on a rotator kept at 4°C inside the BSL3 facility. We used a Tube Rotator and Rotisseries from VWR, Cat#10136-084 (Figure 1), which we placed in a Standard Series Freestanding Undercounter Refrigerator and Freezer from VWR, Cat# 10819-894.

Buffers

Note: All salt buffers should be HPLC pure.

Lysis buffer base

Prepare 2 × 50 mL conical tubes of Lysis Buffer Base. This amount will be sufficient for making β-d-glucopyranoside Stock Buffer and Lysis Wash Buffer (-PI).

Reagent	Final concentration	Amount
1 M Tris, pH 8.0	20 mM	1 mL
5 M NaCl	100 mM	1 mL
1 M MgCl2	6 mM	300 µL
0.5 M EDTA	1 mM	100 µL
ddH_2O	N/A	47.6 mL
Total	**N/A**	**50 mL**

Note: Lysis Buffer Base can be stored in −20°C for 3 months.

⚠ CRITICAL: Mg2+ is an essential cofactor for Benzonase activity. HLA-IP protocols employing sonication to shear DNA may utilize Mg2+ free lysis buffers. Ensure using $MgCl_2$ containing lysis buffer when Benzonase digestion is the method of choice for eliminating DNA (see step 2 of the protocol for information on degradation of nucleic acids using Benzonase).

Octyl β-d-glucopyranoside stock solution

Dissolve 5 g of β-d-glucopyranoside (see key resources table) by adding 17 mL of Lysis Buffer Base directly to the container and mix by vortexing every 10 min until powder is completely dissolved (~30 min).

Note: Stock solution can be stored in 4°C for a few weeks.

500 mM iodoacetamide aliquots

Dissolve 56 g of Iodoacetamide (a single vial, see key reagents table) in 605 µL ddH_2O for a final concentration of 500 mM and aliquot to 50 µL aliquots in light-sensitive microcentrifuge tubes.

⚠ CRITICAL: Iodoacetamide is sensitive to light. Use light-sensitive microcentrifuge tubes to store aliquots.

Note: Iodoacetamide aliquots can be stored at −20°C and should maximum be thawed twice before being discarded.

Lysis wash buffer (-PI)

Prepare Lysis Wash Buffer (-PI) in a 50 mL conical tube. Half of the amount will be used for washing beads after the immunoprecipitation step and half will be used to make Lysis Buffer (+PI). After adding all reagents, place the 50 mL tube in a 37°C water bath for at least 30 min and invert the tube gently to allow the detergents to mix well.

Reagent	Final concentration	Amount
Triton X-100	1.5%	750 µL
Octyl β-d-glucopyranoside stock solution	60 mM	3 mL
500 mM Iodoacetamide	0.2 mM	20 µL
Lysis Buffer Base	N/A	46.230 mL
Total	**N/A**	**50 mL**

Note: Triton X-100 is highly viscous. Cut off the tip of the pipette with disposable scalpel and transfer an accurate amount.

Note: When mixing reagents, invert the tube gently to avoid bubbles.

Note: The 25 mL of the lysis wash buffer used for washing beads (step 5 of the protocol) can be aliquoted to three 15 mL conical tubes, each containing ~8 mL, and can be stored in −20°C for one month. Cover tubes with aluminum foil to protect the content from light since the lysis wash buffer contains light sensitive Iodoacetamide.

Lysis buffer (+PI)

Prepare Lysis Buffer (+PI) using the remaining Lysis Wash Buffer (-PI) after removing 25 mL for beads washing.

- Dissolve 1 tablet of 50× cOmplete™, EDTA-free Protease Inhibitor Cocktail in 1,000 µL ddH_2O and add 500 µL to 24.5 mL of the lysis buffer to achieve 1× concentration.
- Aliquot the 25 mL into three 15 mL conical tubes, each containing ~ 8 mL. Tubes can be frozen in −20°C for 1 month before proceeding to the next step. Cover tubes with aluminum foil to protect from light as the buffer contains light-sensitive Iodoacetamide.
- On the day of HLA-I immunoprecipitation, add PMSF to the 8 mL lysis buffer to a final concentration of 1 mM.

Note: PMSF should be added fresh on the day of immunoprecipitation.

⚠ CRITICAL: PMSF powder is toxic. Use a biosafety cabinet and appropriate personal protective equipment (PPE) when working with PMSF powder.

Wash Buffer Base

Reagent	Final concentration	Amount
1 M Tris, pH 8.0	20 mM	1 mL
5 M NaCl	100 mM	1 mL
0.5 M EDTA	1 mM	100 µL
ddH_2O	N/A	47.9 mL
Total	**N/A**	**50 mL**

Note: Wash Buffer Base can be stored at −20°C for 3 months.

Complete Wash Buffer

Reagent	Final concentration	Amount
Octyl β-d-glucopyranoside stock solution	60 mM	1.5 mL
500 mM Iodoacetamide	0.2 mM	10 μL
Wash Buffer Base	N/A	23.5 mL
Total	**N/A**	**25 mL**

Note: Discard Complete Wash Buffer at the end of the experiment.

20 mM Tris Wash Buffer

Reagent	Final concentration	Amount
1 M Tris, pH 8.0	20 mM	500 μL
ddH_2O	N/A	24.5 mL
Total	**20 mM**	**25 mL**

Note: Discard 20 mM Tris Wash Buffer at the end of the experiment.

STEP-BY-STEP METHOD DETAILS

Preparing cell lysate

Timing: 3 h (in BSL3)

The following steps describe how to lyse infected cells in dishes, degrade nucleic acids, and prepare clear lysates for immunoprecipitation of HLA-I complexes. DNA shearing is a critical step in the immunoprecipitation protocol. Scientists often use sonication, which imposes a risk when working with high-containment pathogens due to aerosol production and potential spread of infectious particles. Thus, we replaced sonication with enzymatic DNA shearing using Benzonase as described before.[2]

1. Lysis of infected cells.
   a. Place 15 cm dishes with infected cells on ice (process 1 dish at a time).
   b. Carefully remove the culture medium and wash cells with 10 mL of ice-cold 1× PBS.
   c. Carefully remove all of the PBS by tilting the dish (any residual amount of PBS will affect the composition of the lysis buffer). Add 2.5 mL of cold Lysis Buffer (+PI).
   d. Scrape cells in the Lysis Buffer using a cell scraper. Transfer to an ice-cold 15 mL conical tube (expected volume is ∼3 mL: 2.5 mL of lysis buffer + cells + residual PBS).
   e. Lyse cells in the other two 15 cm dishes. Collect all lysates in the same 15 mL tube to obtain the total volume of ∼ 9 mL (∼3 mL per dish).
   f. Remove PBS from the 6 "soaked" microcentrifuge tubes. Using a serological pipette, gently mix the ∼9 mL cell lysate 20 times and transfer to the 6 microcentrifuge tubes, ∼1.5 mL per tube.

Note: Depending on the duration of infection and the cell type used, cells may die and/or detach from the dishes (for example, SARS-CoV-2 infected HEK293T cells at 24 hpi). To include floating cells in the experiment, do not discard the media in step 1b, but instead, collect it in a conical tube and spin down at 1,000 × *g* 4°C for 5 min. Remove the supernatant and wash the cell pellet once with ice-cold 1× PBS. Resuspend the cell pellet in the Lysis Buffer that was used to scrape cells from the dishes.

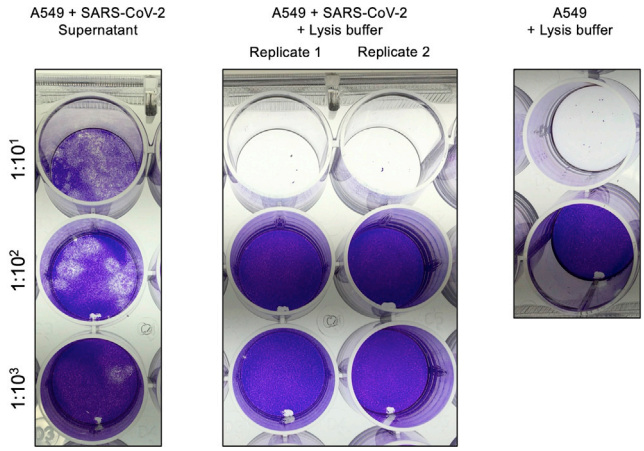

Figure 2. Plaque assay confirming SARS-CoV-2 inactivation after 3 h incubation with lysis buffer containing Triton-X- and benzonase

A549 cells were infected with SARS-CoV-2 at MOI of 3 for 24 h. 10-fold serial dilutions were prepared in Opti-MEM and used to infect Vero cells in a 24-well plate. Comparing plaques in (left) cultured media of infected A549 cells; (middle) SARS-CoV-2 infected A549 cells treated with a lysis buffer containing 1.5% Triton-X and Benzonase for 3 h; and (right) non-infected A549 cells. When adding the 1:10 dilution of the lysis buffer, infected and non-infected cells died immediately due to the relatively high Triton-X concentration. Figure 2 was adapted from Figure S1 in Weingarten-Gabbay et al.[1]

⚠ CRITICAL: Do not use trypsin to detach cells from dishes. Trypsin remnants can interfere with mass spectrometry analysis.

2. Nucleic acid degradation.
   a. Add 1 μL of Benzonase to each of the 6 microcentrifuge tubes containing 1.5 mL of cell lysates.
   b. Incubate tubes on ice for 15 min. Invert tubes every 5 min. Spin down in a pre-cooled swinging-bucket centrifuge for 22 min at maximum speed (4,750 rpm, 4°C) to remove cell debris.
   c. Remove tubes from the centrifuge and keep them on ice while preparing beads and antibodies for the immunoprecipitation step.

Immuno-precipitating HLA-I complexes from cell lysates

Timing: 3.5 h (in BSL3)

The steps below describe the immunoprecipitation of the HLA-peptide complex from infected cell lysates using a pan-HLA-I antibody. Our assays showed that during the 3 h immunoprecipitation step, SARS-CoV-2 is completely inactivated by 1.5% Triton-X and Benzonase present in the lysis buffer (Figure 2).

3. Preparation of antibody-bead mixture.
   a. Centrifuge the microcentrifuge tubes containing washed beads prepared in step 12f of the "before you begin" section at 4,000 rpm for 1 min at 4°C.
   b. Remove the supernatant without disturbing the beads.
   c. Add 50 μL of W6/32 pan-HLA-A/B/C antibody to each tube.
4. Incubation of lysates with beads for immunoprecipitation.
   a. Transfer clear lysates from step 2c to microcentrifuge tubes containing beads and antibodies using a p1000 pipette. When transferring lysates, avoid cell debris at the bottom of the tubes.
   b. Incubate the tubes on a rotisserie-style rotator in a 4°C mini fridge for 3 h. After 3 h, the virus is inactivated, and the immunoprecipitated complexes are safe to be removed from BSL3.

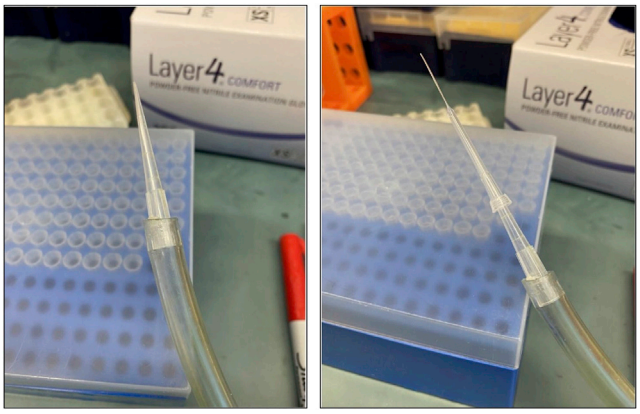

Figure 3. Representative images depicting vacuum setup for bead washing and drying

(Left) For the bead washing setup, an unfiltered 20 μL pipette tip was attached to a suction tube. (Right) For the bead drying setup, a 0.5 μL–10 μL gel loading pipet tip (See key resources table) was attached to the unfiltered 20 μL pipette tip.

⚠ CRITICAL: At the end of this step, both SARS-CoV-2 and VSV were found to be completely inactivated when tested by the standard plaque assay. When working with a new enveloped virus, inactivation should be confirmed before removing samples from BSL3 (or other specified containment level) for the remaining steps of the protocol.

Note: When transferring the IP tubes from BSL3 to BSL1, use an ice bucket or a pre-chilled cold block at 4°C.

Washing beads after HLA-I immunoprecipitation

Timing: 2 h (in BSL1)

After immunoprecipitation is complete, beads are thoroughly washed to remove unbound proteins and antibodies, as well as enzymes, detergents and salts contained in the lysis buffer. Buffers are gradually exchanged during the course of 9 washing steps until the lysis buffer is replaced with Tris 20 mM. At the end of these steps, samples are ready for peptide elution and mass spectrometry analysis.

Note: All wash steps were done using vacuum pipette (Figure 3). Centrifugation was performed at 2,500 rcf for 1 min at 4°C.

Note: All wash buffers should be ice-cold.

5. Washing beads once with Lysis Wash Buffer.
   a. Spin down beads for 1 min, 2,500 rcf at 4°C.
   b. Aspirate supernatant using vacuum pipette. Do not disturb the bead pellet.
   c. Add 1 mL of Lysis Wash Buffer.
   d. Vortex for 10 s and repeat centrifugation.

   Optional: Unbound cell lysates can be used for whole proteome analysis of infected cells. In that case, instead of aspirating lysates, transfer them to 6 labeled ice-cold microcentrifuge tubes using a p1000 pipette and store at −80°C.

6. Washing beads 4 times with Complete Wash Buffer.
   a. Aspirate the supernatant. Do not disturb the bead pellet.

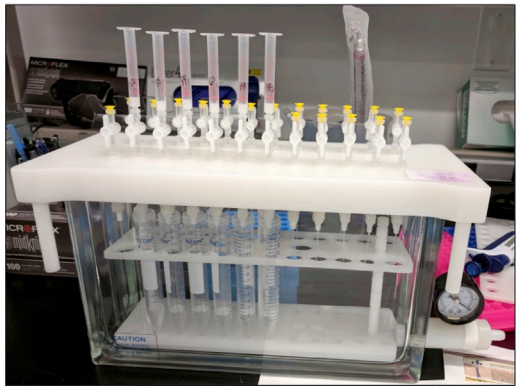

Figure 4. Setting 50 mg tC18 Sep-Pak cartridges on a vacuum manifold for HLA-I peptides elution from beads

b. Add 1 mL of Complete Wash Buffer.
c. Vortex for 10 s and repeat centrifugation.

7. Washing beads 4 times with 20 mM Tris Buffer.
   a. Aspirate the supernatant.
   b. Add 1 mL of 20 mM Tris Buffer.
   c. Vortex for 10 s and repeat centrifugation.
8. Drying beads.
   a. Upon completion of final wash, aspirate supernatant using vacuum pipette without disturbing the beads pellet.
   b. Remove the residual volume of the buffer using gel loading tips (Refer to key resources table). Beads will transform from translucent to white and should appear like salt crystals.
   c. Store the dry beads at −80°C.

Note: When drying the pellet, the skinny tips can touch the beads to adequately dry the beads.

Note: HLA-I peptide complexes are stable at −80°C for 3–6 months.

Purifying peptides from beads for mass spectrometry analysis

Timing: 1–2 h (in BSL1)

Peptides are eluted from dry beads using 50 mg tC18 Sep-Pak cartridge and a vacuum manifold (Figure 4). At the end of these steps, samples are ready for mass spectrometry analysis.

9. Preparing 50 mg tC18 Sep-Pak cartridge for peptides binding.
   a. Rinse cartridge with 200 µL 100% Methanol two times.
   b. Rinse cartridge with 100 µL 99% acetonitrile (ACN) /0.1% formic acid (FA) one time.
   c. Rinse cartridge with 500 µL 1% formic acid four times.
10. Transferring beads from IP tubes to a 50 mg tC18 Sep-Pak cartridge.
   a. Resuspend dry beads in each IP tube with 200 µL 3% ACN/5% FA.
   b. Spike in 1 µL 100 fmol/µL iRT peptides standards into each sample (1 µL of 1/10 iRT dilution).
   c. Transfer beads from the 6 IP tubes to a single 50 mg tC18 Sep-Pak cartridge.
   d. Rinse original IP tubes with 200 µL 1% FA and transfer to the cartridge to collect all beads.
11. Eluting peptides from beads to the cartridge.
   a. Add 200 µL 10% Acetic acid (AcOH) to the cartridge and incubate for 5 min before discarding the buffer using the vaccum. Repeat this step two more times.
   b. Wash the cartridge with 500 µL 1% FA four times.

Table 1. Summary of the number of cells, beads, antibody and resolved HLA-I peptides

Cell line	Number of cells	Number of IPs	Gamma-bind plus sepharose beads (per IP)	W6/32 antibody (per IP)	Total peptides	Viral peptides
A549/ACE2/TMPRSS2 + SARS-CoV-2 24 h	4.5*10^7	6	30 µL	10 µg (50 µL)	6,372	13
HEK293T/ACE2/TMPRSS2 + SARS-CoV-2 24 h	6*10^7	6	30 µL	10 µg (50 µL)	1,336[a]	8

[a]SARS-CoV-2 infection resulted in ~50% cell death of the infected HEK293T cells, reducing the total amount of resolved HLA-I peptides.

12. Eluting peptides from cartridge to a new collection tube.
    a. Place a new 2 mL Eppendorf collection tube.
    b. Rinse cartridge with 250 µL 15% ACN/1% FA once (Elution 1).
    c. Rinse cartridge with 250 µL 50% ACN/1% FA two times (Elution 2).
    d. Dry eluted peptides using SpeedVac and store at −80°C until mass spectrometry analysis.
    e. Resuspend peptides in 5 µL 3% ACN/5% FA and inject 4 µL onto mass spectrometer.

EXPECTED OUTCOMES

HLA-I peptides were analyzed in the mass spectrometry facility as described by Klaeger et al.[10]. Samples prepared with this protocol yielded thousands of peptides (Table 1) in the expected length of 8–11 amino acids and sequence motifs matching the expressed HLA-I alleles in A549 and HEK293T cells.[1] Incubating the cell lysates for 3 h at 4°C (step 4 of the protocol) led to complete inactivation of SARS-CoV-2 as determined by a standard plaque assay (Figure 2).

LIMITATIONS

Since virus inactivation is achieved through detergent-mediated disruption of viral envelope, this protocol does not inactivate non-enveloped viruses. In addition, the relatively large number of infected cells required to obtain sufficient input material for mass spectrometry (45–60*10^6 cells per experiment) imposes a challenge when working with primary cells and cell lines with slow growth rate.

TROUBLESHOOTING

Problem 1

Virus is not fully inactivated after 3 h incubation of cell lysates at 4°C (step 4 of the protocol).

Potential solution

Increase the volume of the lysis buffer and/or incubation time. When calibrating the protocol for VSV, we noticed that adding 350 µL lysis buffer to a 10 cm dish (the ratio previously used for HLA-I peptidome experiments in uninfected cells) was not sufficient to inactivate the virus (Table 2). Increasing the volume by ~3-fold to 1 mL per 10 cm dish resulted in complete inactivation. The same amount was sufficient to inactivate SARS-CoV-2 as well (Figure 2), however, it is possible that other viruses will require more stringent conditions.

Problem 2

Beads do not form a proper pellet after the 3 h immunoprecipitation (step 5 of the protocol).

Potential solution

Ensure that the lysis buffer contains a sufficient amount of Mg2+ (see Lysis Buffer Base composition in materials and equipment). Undigested DNA can interfere with HLA immunoprecipitation and the quality of the results. This protocol does not use sonication for DNA shearing and thus relies on the enzymatic activity of Benzonase. Mg2+ is an essential cofactor of Benzonase and using a $MgCl_2$ containing buffer is critical for the success of the protocol.[2] We highly encourage readers to use the exact composition of all buffers as provided in the materials and equipment section and to not

Table 2. Calibrating VSV inactivation in infected HEK239T cells

Condition	Lysates treatment	Estimated PFU/mL
Negative cntrl	Non-infected cells + 350 µL Lysis buffer	0
Positive cntrl	Infected cells + 350 µL PBS	Too many to count
Condition #1	Infected cells + 350 µL lysis buffer 4c for 3 h	4.00E+04
Condition #2	Infected cells +1,000 µL lysis buffer 4c for 3 h	0

HEK239T cells were infected with VSV-eGFP (Indiana strain[11]) at an MOI of 1. After 24 h, infected cells were harvested in a lysis buffer or PBS. The amount of added buffer and incubation time are indicated in the table. Cell lysates were then diluted 1:100 and 1:1,000 in serum-free media and added to fully confluent Vero cells in a 6-well plate. After 1 h incubation at 37°C, lysates were removed and methocel plaquing media was added. 24 h post infection, cells were stained in methylene blue for 30 min at room temperature and VSV plaques were counted by eye. The computed plaque forming unit (PFU)/mL of each tested condition are listed in the table.

combine with buffers from other protocols. Furthermore, peptides can be subjected to a second desalting step before LC-MS/MS analysis if contamination is visible.

Problem 3

No HLA-I peptides are detected in mass spectrometry (expected outcomes section).

Potential solution

Failing to detect HLA-I peptides after immunoprecipitation of the HLA-I complex can be a result of low HLA-I expression in the tested cell line. To ensure proper expression of the HLA-I complex on the cell surface, flow cytometry analysis can be performed with a specific fluorophore-conjugated pan-HLA-I antibody (Figure 5).

Problem 4

Viral peptides are not observed in the pool of HLA-I peptides (expected outcomes section).

Potential solution

Absence of viral sequences from the pool of the observed HLA-I peptides can result from low abundance of viral proteins or from missing the peak of viral peptides presentation. There are few potential solutions depending on the source of the problem:

- Ensure that the chosen cells are properly infected with the virus using appropriate methods to quantify virus infectivity such as plaque assay, tissue culture infectious dose ($TCID_{50}$) etc.
- Consider increasing the MOI to achieve a higher virus copy number per cell.
- Consider performing the HLA-I immunoprecipitation at a different time point. Notably, a few studies, including ours, found that some viral HLA-I peptides peak as early as 3–6 h post infection.[1,8,9] Profiling HLA-I peptidome at later time points can miss the detection window of early presented viral peptides.
- Increase the number of infected cells used for the immunoprecipitation in order to increase the coverage of detected peptides.

Problem 5

Massive cell death due to virus infection leading to floating cells in the dish. Performing on-plate cell lysis and discarding the media under these conditions (step 1 of the protocol) will result in losing a substantial number of cells, thereby lowering the yield of HLA-I peptides.

Potential solution

To reduce the fraction of dead cells, consider infecting cells with lower MOI or performing the HLA-I immunoprecipitation at an earlier time point post infection. If you wish to include floating cells in the HLA-I peptidome analysis, collect the culture media in a conical tube and pellet down floating cells by centrifugation at 1,000 × *g* 4°C for 5 min. Remove supernatant, and wash the cell pellet once with

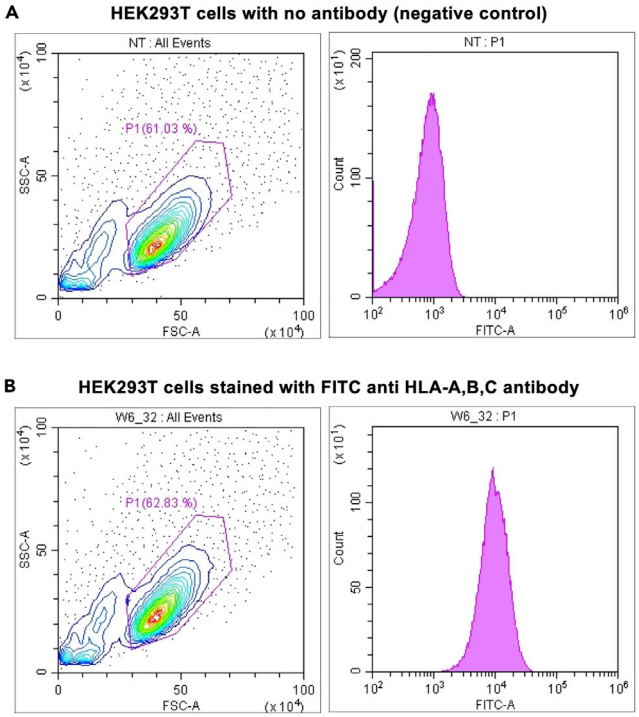

Figure 5. Flow cytometry analysis of HLA-I expression on the surface of HEK293T cells

1*10^6 HEK293T cells were harvested using trypsin, washed once with 1× PBS and resuspended in 100 μL 1× PBS. Cells were stained with 3 μL of anti-human HLA-A,B,C antibody (FITC-W6/32) and incubated for 25 min at room temperature. After staining, cells were washed once with PBS, resuspended in 700 μL PBS and analyzed in flow cytometry.

(A) Non-stained cells were used as negative control to determine autofluorescence background. Population gating using FSC and SSC channels is shown in the left panel and FITC intensity levels are shown on the right.

(B) Similar to (A) but for cells stained with pan-HLA-I antibody showing HLA-I expression above background.

ice cold 1× PBS. Perform on-plate cell lysis for the remaining attached cells as described in step 1 and use the same cell lysate to resuspend the pellet of floating cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shira Weingarten-Gabbay (shirawg@broadinstitute.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets or code.

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AUTHOR CONTRIBUTIONS

S.W.-G. conceptualized the study. S.W.-G., L.R.P., D.-Y.C., S.K., and N.L.W. performed experiments. H.B.T., D.B.K., S.A.C., and J.G.A. contributed protocols. M.S. and P.C.S. supervised experiments. S.W.-G., L.R.P., D.-Y.C., M.S., and P.C.S. wrote the manuscript with comments from all authors.

DECLARATION OF INTERESTS

S.W.-G., S.K., S.A.C., J.G.A., M.S., and P.C.S. are named co-inventors on a patent application related to this manuscript filed by The Broad Institute that is being made available in accordance with the COVID-19 technology licensing framework to maximize access to university innovations. N.L.W. is a consultant for Carver Biosciences. D.B.K. own equity in Affimed N.V., Agenus, Armata Pharmaceuticals, Breakbio, BioMarin Pharmaceutical, Celldex Therapeutics, Clovis Oncology, Editas Medicine, Exelixis, Gilead Sciences, Immunitybio, ImmunoGen, IMV, Lexicon Pharmaceuticals, Moderna, Neoleukin Therapeutics, Regeneron Pharmaceuticals, and Sesen Bio. BeiGene, a Chinese biotech company, supported unrelated SARS COV-2 research at TIGL. S.A.C. is a member of the scientific advisory boards of Kymera, PrognomIQ, PTM BioLabs, and Seer and an ad hoc scientific advisor to Pfizer and Biogen. J.G.A. is a past employee and shareholder of Neon Therapeutics, Inc. (now BioNTech US). P.C.S. is a co-founder of and consultant to Sherlock Biosciences and Delve Biosciences and a board member of Danaher Corporation and holds equity in the companies.

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