Cancer exome-based identification of tumor neo-antigens using mass spectrometry

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Running Title: Identification of neo-antigens using HLA peptidomics

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Abstract

Neo-antigens expressed on tumors are targets for development of cancer immunotherapy strategies. Use of prediction algorithms to identify neo-antigens yield a significant number of peptides that must be validated in laborious and time-consuming methods; many prove to be false positive identifications. The use of HLA peptidomics allows the isolation of the HLA-peptide complexes directly from cells and can be done on fresh tumor, patient-derived xerographs, or cell lines when the tissue sample is limited. This method can be used to identify both HLA class I and HLA class II or any different MHC from different species. Here we describe the steps to create the immune-affinity columns used from the process, the immunoprecipitation procedure, and also the isolation of the peptides that will be analyzed by mass spectrometry.

Keywords

Neo-antigens, HLA peptidomics, MHC peptides, exome-sequencing, mass spectrometry

1 INTRODUCTION

Until recently, most immunotherapy strategies targeted known tumor-associated antigens and, in melanoma, the melanocyte differentiation antigens, but these therapies had limited success and often caused adverse immune reactions due to the use of self-antigens [1]. It is now known that the majority of the immune response against tumors results from the presentation of mutated antigens, which are called neo-antigens [2,3]. Recent clinical studies using neo-antigen peptides and RNA vaccines [4,5] and neo-antigen-enriched tumor infiltrating lymphocyte (TIL) transfer [6] showed promising results in patients.

There are two main strategies to identify neo-antigens, the first is based on algorithms that predict the binding of possible neo-antigens to the cell's HLA alleles followed by experimental validation of the immunogenicity of the identified peptides [7]. The second is the direct immune-purification of the HLA-peptide complexes from cells and identification of the peptides sequences by mass spectrometry; this strategy is called HLA peptidomics [8-11]. The main disadvantage of the prediction algorithm strategy is that it yields many false-positive peptides, and, therefore, peptides must be validated using laborious and time-consuming methods such as tandem mini-gens [12,5,4,13], synthetic peptides [14], or HLA-tetramers/multimers [15]. Moreover, this strategy can only identify neo-antigens to which the patient already has prior immunity, since the validation is based on confirmation of immunogenicity of the peptide. It can be that these antigens are no longer presented by the tumor and therefore no longer clinically relevant. In contrast, HLA peptidomics identifies the neo-antigens that are actually presented by the cells. Even if the patient does not have prior immunity against a

particulate neo-antigen, engineered T cells that express the TCR against the antigen can be used to treat the patient [16].

To employ the HLA peptidomics approach, the tumor sample and matched normal sample should first be analyzed by whole exome or genome sequencing to identify the somatic mutations in the tumor sample. The tumor cells or tissue are then lysed and used for the immunoprecipitation of the HLA-peptide complexes. After isolation from the HLA molecules, the peptides are sequenced by mass spectrometry, and the data are then analyzed by comparison of the complete human proteome and the patient specific-mutated proteins list to identify the neo-antigens. In this chapter, we will describe all the steps from the purification of the antibodies to the preparation of the affinity column beads and later the purification of the MHC-peptide complexes and isolation and analysis of the eluted peptides in the mass spectrometer.

2 MATERIALS

Prepare all solutions in sections 2.2 - 2.3 with water purified by double deionization. Prepare all solutions in sections 2.4 - 2.7 with HPLC-grade water, keep all solutions in glass bottles (not plastic), and do not use autoclaved tips or bottles.

2.1 Hybridoma cells and antibody production

1. Hybridoma cells: Cells should be selected to correspond to the species from which the MHC is purified. For human samples use HB-95 hybridoma cells (ATCC) to produce pan-anti-HLA-A, B, C antibodies (W6/32) and HB-145 hybridoma cells (ATCC) to produce anti-HLA-DR, DQ, DP antibodies (IVA12). For murine samples, there are a variety of hybridoma cells that produce antibodies against specific MHC alleles.
2. Cell culture: Cells are grown in a 5% CO_2 humidified incubator in flasks (horizontally) containing filtered DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, and 100 U/ml pen-strep.
3. CELLine 350 bioreactor flask for suspension cell culture (Argos).

2.2 Antibody purification

1. Protein A Plus Agarose (Thermo-Fisher Scientific) or Protein G Plus Agarose (Thermo-Fisher Scientific). Protein A or G should be used according to the IgG type. For W6/32, use protein A and for IVA12 use protein G.
2. Bio-Spin chromatography column (BIO-RAD).
3. Elution buffer: 0.1 N acetic acid, pH 3.
4. Equilibration buffer: 100 mM Tris-HCl, pH 8.5.

5. Washing buffer: phosphate-buffered saline (PBS).
6. Neutralizing buffer: 1 M Na_2HPO_4, pH 9.
7. Storage buffer: PBS containing 0.02% sodium azide.

2.3 Construction of anti-MHC beads

1. Protein A Plus Agarose (Thermo-Fisher Scientific).
2. Bio-Spin chromatography column (BIO-RAD).
3. Elution buffer: 0.1 N acetic acid, pH 3.
4. Equilibration buffer: 100 mM Tris-HCl, pH 8.5.
5. Washing buffer: 0.2 M sodium borate, pH 9.
6. Cross-linking reagent: dimethyl pimelimidate.
7. Washing buffer: 0.2 M ethanolamine-HCl, pH 8.
8. Storage buffer: PBS containing 0.02% sodium azide.

2.4 Lysis of cell pellet or fresh tumor

It is important to prepare all solutions using HPLC-grade water.

1. Lysis buffer: Prepare fresh lysis buffer before every immunoprecipitation. The buffer is 0.25% sodium deoxycholate, 0.2 mM iodoacetamide (prepare solution with 33 mg in 1 ml PBS and add 1 µl for every 1 ml of lysis buffer), 1 mM EDTA (prepare solution of 0.5 M EDTA and add 2 µl for every 1 ml of lysis buffer), 1% octyl-β-D glucopyransoside, 1:200 protease inhibitor (SIGMA) and 10 µl of 0.1 M phenylmethylsulfonyl fluoride in cold PBS.
2. Cell strainer 40 µm.

2.5 Pre-clearing column

1. Bio-Spin chromatography column (BIO-RAD).
2. Elution buffer: 0.1 N acetic acid, pH 3.
3. Washing buffer: PBS.

2.6 Immunoprecipitation of MHC-peptide complexes

It is important to prepare all solutions using HPLC-grade water.

1. Tip column (TopTip, Glygen, TT3EMT).
2. Syringe (Ritter ritips, 12.5 ml).
3. Elution buffer: 1% trifluoroacetic acid (TFA).
4. Equilibration buffer: 20 mM Tris-HCl, pH 8.
5. Pre-elution buffer: 0.1 N acetic acid, pH 3.
6. Lysis buffer (as in 2.4.1).
7. 400 mM NaCl, 20 mM Tris-HCl, pH 8.

2.7 Isolation of HLA peptides

It is important to prepare all solutions using HPLC-grade water.

1. Ultra-Micro Tip Column C18 tip (Harvard Apparatus, 74-7106).
2. 80% Acetonitrile (ACN) 0.1% TFA.
3. 30% ACN, 0.1% TFA.
4. 0.1% TFA.

2.8 Mass spectrometry instrumentation

Chromatography is performed with the UltiMate 3000 RSLCnano-capillary UHPLC system (Thermo Fisher Scientific), which is coupled by electrospray to tandem mass spectrometry on Q-Exactive-Plus (Thermo Fisher Scientific). Samples are resolved on capillary reversed phase chromatography on 0.075x300 mm laser-pulled capillaries, self-packed with C18 reversed-phase 3.5-µm beads (Reprosil-C18-Aqua, Dr. Maisch GmbH) [17].

3 METHODS

3.1 Cultivation of hybridomas in the CELLine bioreactors

The CELLine bioreactor is designed to enable culture of cells at high density, so that large amounts of secreted proteins, such as antibodies, can be produced [18].

1. Grow about $5x10^7$ hybridoma cells.
2. Collect the cells and centrifuge at 300g for 5 min.
3. Re-suspend the cells in 5 ml complete DMEM (as described in section 2.1.2) and seed into the cell compartment.
4. Add 350 ml of complete DMEM into the medium compartment.
5. Incubate the bioreactor 10 days in a 5% CO_2 humidified incubator.
6. Harvest the conditioned medium containing the antibody. Centrifuge at 300g for 5 min and keep the supernatant. The cells can be re-seeded for another cycle or fresh cells can be cultivated for another cycle of 10 days.

7. Centrifuge the supernatant at 1800g for 5 min to remove cell debris and store at 4ºC until ready to purify the antibodies.

3.2 Purification of antibodies

1. Add 300 µl of protein A (for W6/32) or protein G (for IVA12) Sepharose beads to a Bio-Spin chromatography column. Let the preservation solution flow through; do not let the beads dry at any step.
2. Perform a pre-elution with one column volume of 0.1 N acetic acid, pH 3.
3. Equilibrate the column with 100 mM Tris-HCl, pH 8.5.
4. Load the supernatant containing the W6/32 or IVA12 antibody onto the column and let it flow through the beads. Collect an aliquot of the supernatant before loading it to the column. This will be compared to the flow through in order to evaluate the efficiency of the purification.
5. Wash with 10 column volumes of cold PBS.
6. Perform 5 elutions with 250 µl 0.1 N acetic acid, pH 3 into microfuge tubes containing 90 µl of 1 M Na_2HPO_4, pH 9. Prior to the first elution, mix 250 µl of 0.1 N acetic acid, pH 3 with 90 µl of 1 M Na_2HPO_4. If the solution is not pH 8, change slightly the amount of 1 M Na_2HPO_4 used in elutions to obtain a pH of 8.
7. Equilibrate column with 100 mM Tris-HCl, pH 8, and then store the column in PBS containing 0.02% sodium azide at 4 ºC (*see* **Note 1**).
8. Run samples of the supernatant and flow through on 10% acrylamide SDS-PAGE and then stain with Coomassie blue to evaluate the efficiency of the purification. You should see a decrease in the amount of antibody in the flow through or no band. Also run small aliquot (~0.5%) of the antibody elutions to evaluate their purity and quantity. The quantity of the antibody can be evaluated

by running on the same gel known amounts of BSA and comparing the size of the band (Figure 1) (*see* **Note 2**).

3.3 Construction of covalently bound Protein A – antibody beads

1. Add 1 ml of protein A Sepharose beads to Bio-Spin chromatography column. Let the preservation solution flow through, but do not let the beads dry at any step.
2. Perform a pre-elution with one column volume of 0.1 N acetic acid, pH 3.
3. Equilibrate with 100 mM Tris-HCl, pH 8.5.
4. Add 2 mg of purified antibodies to the beads and incubate with rotation for 1 h at room temperature. Make sure that the antibody solution covers the beads.
5. Let the solution flow through, and wash the column with 10 column volumes of 0.2 M sodium borate, pH 9.
6. Resuspend the beads in 2 ml of 0.2 M sodium borate, pH 9 and remove 40 µl of beads for later analysis.
7. Add dimethyl pimelimidate to a final concentration of 20 µM and incubate with rotation for 30 min.
8. Save 12 µl of the beads for later analysis and let the rest of the solution flow through.
9. Perform the following steps in a chemical fume hood. Wash the column with 0.2 M ethanolamine-HCl, pH 8.
10. Add 5 ml of 0.2 M ethanolamine-HCl, pH 8 and incubate with rotation for 2 h.
11. Wash with 10 ml of 100 mM Tris-HCl, pH 8.0 and store at 4ºC with 0.02% sodium azide in PBS.
12. Run aliquots of the samples before and after cross-linking of the antibody on 10% acrylamide SDS-PAGE and then stain with Coomassie (*see* **Note 3**).

3.4 Evaluation of the Protein A – antibody bead binding efficiency

It is recommended that the immunoprecipitation ability of the beads be tested using a small aliquot of the beads and an aliquot of cells. Perform the purification as below and then run 10% of the elution on 10% acrylamide SDS-PAGE and then stain with Coomassie (*see* **Note 4**).

3.5 Collection of cell pellet for MHC-peptide complex immunoprecipitation

Cultured cell line:

1. Grow cells as recommended to reach the required amount of $2x10^8$ cells.
2. Use trypsin to detach adherent cells; collect cells into tubes.
3. Centrifuge at 300g for 5 min.
4. Remove supernatant and re-suspend all cell pellets in a total of 10 ml PBS in one 15-ml conical tube.
5. Centrifuge at 300g for 5 min.
6. Remove supernatant (make sure not to touch the cells) and immediately freeze in liquid nitrogen and store at -80ºC or continue to immunoprecipitation step directly.

Fresh tumor samples:

Fresh tumor samples should be frozen in liquid nitrogen and stored at -80ºC.

3.6 Lysis of the cell pellet or fresh tumor sample

3.6.1 Lysis of cells in a pellet:

1. Thaw the cell pellet on ice. Add lysis buffer according to the cell number and pipet thoroughly to homogenize. Use 4 ml of lysis buffer for $2x10^8$ cells (*see* **Note 5**).

3.6.2 Lysis of tissue:

1. Weigh the tumor sample. Use about 1 ml of lysis buffer for every 0.1 g of tissue sample.
2. For the dissociation of the tumor, insert a cell strainer (40 μm) into a 3.5-cm plate. Put this plate into a 6-cm plate without the covers and put it on ice.
3. Cut the tissue to small pieces with scalpel and add the pieces to the strainer with the lysis buffer. Use the rubber piston of a 10-ml syringe to squeeze the tissue through the strainer.

For cells or tissue:

1. Collect the lysate including any tissue that did not completely dissociate into a 15-ml tube and incubate with rotation for 1 h at 4˚C.
2. Centrifuge the lysate at 48000 *g* for 45 min at 4˚C. Collect the supernatant into a clean tube (*see* **Note 6**).

3.7 Pre-clearing column

1. Wash Bio-Spin chromatography column with 2 ml of 0.1 N acetic acid, pH 3.
2. Wash with 5 ml of PBS.
3. Add 200 ml of protein A beads and let the preservation solution flow through (*see* **Note 7**).
4. Perform a pre-elution with one column volume of 0.1 N acetic, acid pH 3.

5. Equilibrate with PBS.
6. Add the lysate and collect the flow through.
7. Take a small aliquot (~30 µl) of the lysate as input for later analysis.

3.8 Immunoprecipitation of MHC-peptide complexes

1. Wash a tip column with 1% TFA.
2. Wash the tip with 20 mM Tris-HCl, pH 8.
3. Add 50 µl of the beads prepared in section 3.3 to the tip column and let the preservation solution flow through, but do not let the beads dry at any step.
4. Perform a pre-elution with one column volume of 0.1 N acetic acid, pH 3.
5. Equilibrate with 20 mM Tris-HCl, pH 8.
6. Connect a cylinder of a 10-ml syringe to the tip column to enable the loading of the sample into the tip. Add the cleared lysate to the column using a glass Pasteur pipet with a long and thin neck; fill first the tip column and then add the rest of the volume to the connected cylinder. Make sure there are not air bubbles between the two.
7. Let the lysate flow through the column and collect some of the flow through for later analysis (~30 µl) (*see* **Note 8**).
8. Wash the tip column with 2 ml of lysis buffer (*see* **Note 9**).
9. Wash the tip column with 4 ml of 400 mM NaCl, 20 mM Tris-HCl, pH 8.
10. Wash the tip column with 2 ml of 20 mM Tris-HCl, pH 8.
11. Elute with 250 µl of 1% TFA.

3.9 Isolation of HLA peptides

1. Wash C18 tip with 200 µl of 80% ACN 0.1% TFA four times (*see* **Note 10**).
2. Wash C18 tip with 200 µl of 0.1% TFA four times.
3. Load sample on the C18 tip and collect the flow through, load only 200 µl each time. Repeat the loading of the all sample four times.
4. Wash C18 tip with 200 µl of 0.1% TFA four times.
5. Elute peptides with 100 µl of 30% ACN 0.1% TFA twice into the same tube.
6. Elute proteins (mainly MHC) with 100 µl of 80% ACN 0.1% TFA twice into the same tube.
7. Collect small aliquot (~2%) from the peptide fraction and from the protein fraction for quality and purity analysis.

3.10 Analysis of the quality of purification and purity of the peptide fraction

Run the samples of cleared lysate and flow thorough (~30 µl) and samples from peptide and protein fractions on a 10% acrylamide SDS-PAGE, then transfer to nitrocellulose and perform western blot analysis against the MHC molecule. You should see a decrease in the amount of MHC in the flow through or no band and an intense band in the protein fraction and no band at all in the peptide fraction. If the peptide fraction contains HLA clean it again since HLA will interfere with the mass spectroscopy analysis.

3.11 Identification of eluted MHC peptides using mass spectrometry

1. Dry the MHC peptide fractions by vacuum centrifugation.

2. Resolubilize with 11 µl of 0.1% formic acid and use 5 µl of this solution for the analysis.
3. Use the following settings:
    - Elute the MHC peptides with a linear gradient over 2 h from 5 to 28% ACN with 0.1% formic acid at a flow rate of 0.15 µl/min.
    - Acquire data using a data-dependent “top 10” method, fragmenting the peptides by higher-energy collisional dissociation.
    - A full scan MS spectrum should be acquired at a resolution of 70,000 at 200 m/z with a target value of $3x10^6$ ions. Ions are generally accumulated to an AGC target value of 105 with a maximum injection time of 100 msec.
    - Peptide match option should be set to “Preferred”.
    - Normalized collision energy should be set to 25%.
    - MS/MS resolution should be set to 17,500 at 200 m/z.
    - Fragmented m/z values should be set to be dynamically excluded from further selection for 20 sec.

3.13 Analysis of mass spectrometry data using MaxQuant and identification of neo-antigens

1. Create a FASTA file including the list of all mutated protein sequences according to the whole exome sequencing data of the tumor cells.
2. Use MaxQuant [19] to analyze the mass spectrometry raw data using the mutation database of the cells and the complete human proteome database (UniProt) with the following settings:

- Set N-terminal acetylation (42.010565 Da) and methionine oxidation (15.994915 Da) as variable modifications.
- Set enzyme specificity as unspecific.
- Set peptides FDR to 0.05.
- Enable the “match between runs” option to allow matching of identifications across the samples belonging the same patient.

3. Filter only the peptides from the peptide list that were identified from the mutation site that do not also correspond to another protein.
4. Use NetNHCpan [20,21] to determine the MHC alleles to which the peptide matches.
5. Validate the peptide identification accuracy by comparison of endogenous peptide spectra to synthetic peptide spectra (Figure 2)

4 NOTES

1. Use this column only for specific antibodies to avoid contamination of different antibodies between samples.
2. The elutions should have similar amounts of the heavy (50 kDa) and light (25 kDa) chains. To quantify the amount of antibody in the elution fractions multiply the amount of protein in the BSA band by the signal to obtain the sample dilution factor (total volume of elution/ volume of sample loaded to gel).
3. No heavy-chain of the antibody should appear in the sample after cross-linking.
4. Make sure you see a band for the MHC molecule at the correct size.
5. If the lysate is very dense or if using larger number of cells, add more lysis buffer. Make sure not to dilute too much since it will extend the time of the precipitation step.
6. If the lysate is not clear, centrifuge again at the same speed for 15 min.

7. Do not let the beads dry at any step. If you would like to stop for a brief moment you can cap the column.
8. If you want to use the lysate for immunoprecipitation of MHC class I complexes and later of MHC class II complexes use the flow through immediately after this step for subsequent affinity column. Do not freeze the lysate, it will not be suitable for immunoprecipitation. Similarly, use subsequent affinity columns if you are using antibodies for different alleles on the same lysate. Alternatively, you can make a mix of the different antibodies beads if you do not want to separate the results.
9. To expedite the flow of the lysate or the following washes you can insert the piston of the syringe and apply light pressure. Do not apply too much pressure since it will destroy the beads.
10. All buffers should be prepared fresh.

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FIGURE LEGENDS

Figure 1: Evaluating the amount of purified antibodies. In lanes 1-5 there are the antibody elution bands. There should be a similar amount of the heavy (50kDa) and light (25kDa) chains. Known amounts of BSA are used to quantify the amount of antibody in the elutions.

Figure 2: Tandem mass spectra of endogenous mutant peptides and their corresponding synthetic peptides. The spectra of two neo-antigens that were identified in HLA peptidomics of melanoma cells (12T) (A) KLFEDRVGTIK (B) DANSFLQSV are compared to the spectra of synthetic peptides to validate their identification in the mass spectrometer [8].