Analysis of neoantigen-reactive T cells using the QuickSwitch™ custom tetramer kit




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Neoantigen

Cancer immunotherapy is gaining attention with the advent of immune checkpoint inhibitors such as the anti-PD-1/PD-L1 antibody. The mechanism of action of immune checkpoint inhibitors is to reactivate pre-existing anti-tumor immunity that has been suppressed within tumors. The key player in the re-invigoration of immune response is tumor-specific T cells [1]. Neoantigens derived from mutated gene products in cancer cells have drawn significant interest since neoantigens are indeed the tumor antigens recognized by tumor-specific T cells reactivated by immune checkpoint inhibitors [2].

Cellular proteins are degraded to peptides with 9 to 11 amino acids in length that can bind to the peptide-binding groove of MHC class I molecules. The peptide/MHC complexes are then presented on the cell surface [3]. The T-cell receptor (TCR) recognizes these peptide/MHC complexes.

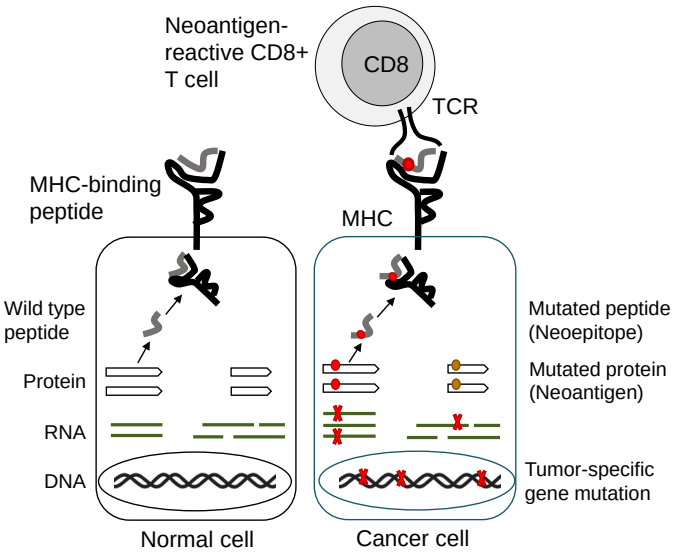


Figure 1. Neoantigens derived from tumor-specific mutant gene products

Normally, immune response against normal cells is inhibited to prevent autoimmunity because the immunological tolerance is induced by negative selection in the thymus. On the other hand, T cells can recognize cancer cells that accumulate many somatic mutations during tumorigenesis express mutated proteins that are not present in normal cells. These mutated proteins that are recognized by T cells are called “neoantigens”. Neoantigens do not induce immune tolerance in the thymus, and therefore are likely to generate strong T cell immune response (highly immunogenic). Mutated peptides that bind MHC class I molecules and are recognized by T cells are called “neoepitopes” (**Figure 1**) [4].

Prediction of neoantigens using next-generation sequencer

Gene mutations in cancer cells are divided into two types; driver mutations that are directly involved in tumorigenesis and cell proliferation, and passenger mutations that are not involved in these processes. While identification of driver mutations that are shared by multiple patients with a certain probability is critical for the development of molecularly targeted drugs, passenger mutations have been ignored for the drug development because most passenger mutations are unique to individual patients. In contrast, neoantigens can be derived from any amino acid mutations, regardless of whether driver or passenger mutations.

With the advent of next-generation sequencers (NGSs), it is now possible to obtain genome information of individual patients. This allows us to predict neoantigens based on the exome and transcriptome data using DNA and RNA extracted from surgical or biopsy specimens of individual patients [5]. The prediction algorithms for neoantigens are not necessarily specialized. Based on the current knowledge of molecular biology and immunology, neoantigen/neoepitope prediction algorithms are created by combining the similar scheme for the identification of driver mutations in the development of molecularly targeted drugs, and

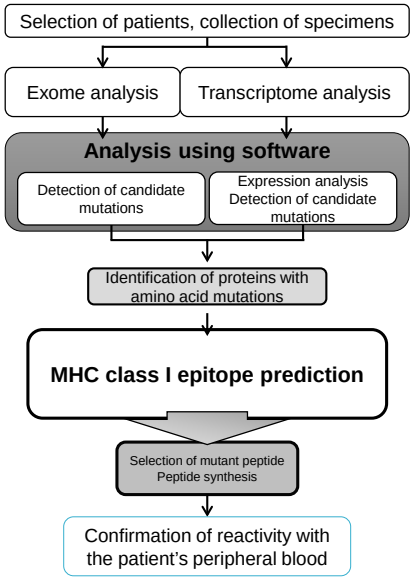


Figure 2. Neoantigen prediction algorithm

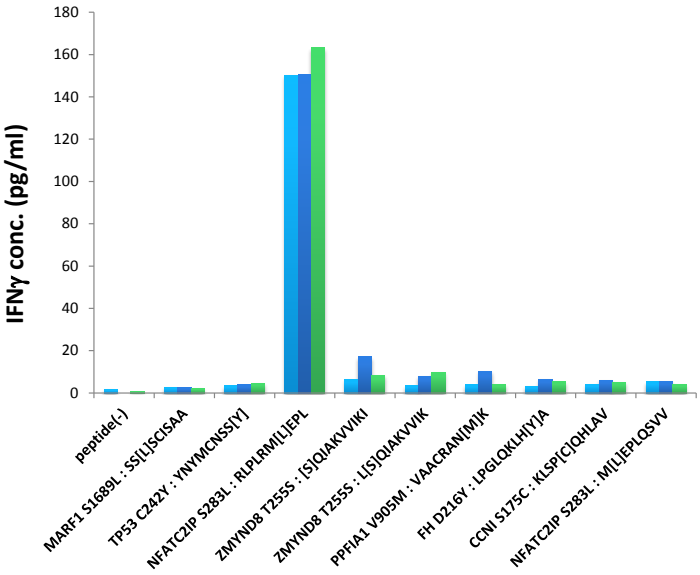


Figure 3. Screening of a neoepitope peptide panel (IFN-γ production was measured by ELISA)

the strategies for the development of peptide cancer vaccines [6]. The algorithm screens mutated gene products with amino acid mutations and produce a list of predicted neoepitopes of 9 to 11 amino acids in length with a high affinity for the patient's MHC.

Screening for neoantigen-reactive T cells

Currently, predictions of neoantigens are merely predictions, and a number of issues need to be addressed including the following: whether lymphocytes reactive to mutant peptides are actually present in the patient's body; if present, to what extent such lymphocytes contribute to tumor-specific immune responses; and how such lymphocytes could be incorporated into treatment. Below is an example of identification of a neoepitope peptide to which the patient's peripheral blood mononuclear cells (PBMCs) actually reacted in an esophagus cancer patient. Based on the estimated MHC class I binding affinity of mutated proteins, the list of neoepitope peptides were prepared, and screened for the reactivity to patient PBMCs.

First, whole-exome sequencing and RNA-Seq were performed. A total of 209 tumor-specific somatic mutations were identified, of which 43 were found to be expressed. Fifty-four neoepitope peptides carrying these mutations were synthesized based on predicted binding affinity for the patient's HLA molecules (HLA-A*02:06, HLA-A*11:01, HLA-B*52:01, HLA-B*54:01, HLA-C*01:02, HLA-C*12:02). The patient's PBMCs were cultured in the presence of each of the peptides and screened for IFN-γ production by ELISA (**Figure 3**). The patient's PBMCs produced IFN-γ in response to the peptide RLPLRM[L]EPL, which carried a mutation of serine to leucine at position 283 of the NFAT C2IP gene. The predicted binding affinity (IC_{50}) of this peptide for HLA-A*02:06 was 163.8 nM, while the IC_{50} of the wild type peptide (RLPLRM[S]EPL) was 894.2 nM.

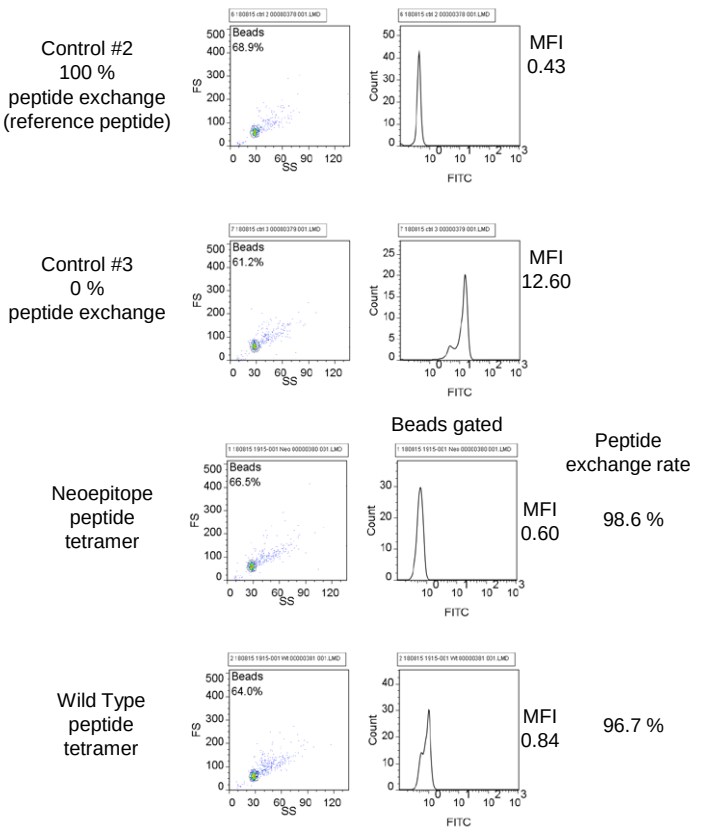
Detection of neoantigen-reactive T cells using QuickSwitch™ custom tetramers

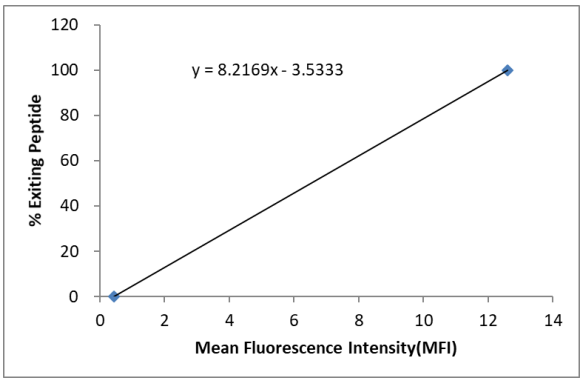
To confirm the presence of neoantigen-reactive T cells in the patient's peripheral blood, the patient's PBMCs were cultured for 24 days with dendritic cells (DCs) pulsed with the neoepitope peptide. After selective expansion, neoantigen-reactive T cells were stained with tetramers. Peptides pre-loaded on MHC tetramers in the QuickSwitch™ custom tetramer kit (MBL International) were exchanged in the laboratory with the neoepitope peptide or a control wild-type peptide just before use. The peptide exchange efficiency was 98.6% with the mutant peptide and 96.7% with the control wild-type peptide (**Figure 4**).

The cultured patient's PBMCs were stained with CD8, CD3, Fixable Viability Dye eFluor™ 780, and the tetramers. The results were that 0.8% of the CD3-positive cells were tetramer-positive, demonstrating the presence of neoantigen-reactive T cells in the patient's peripheral blood (**Figure 5**).

Clonality of neoantigen-reactive T cells

The neoepitope SIF[C]CLDPA, identified in an HLA-A*02-positive esophagus cancer patient, carried a mutation of arginine to cysteine at position 1,049 of the DHX57 gene. T cells that reacted to this epitope were cultured from PBMCs of a healthy donor who had the same HLA-A*02. Since the occurrence of neoantigen-reactive T cells is extremely low in healthy individuals, PBMCs were stimulated twice with the peptide to expand the T cells before detection with the tetramer. The detection was performed using QuickSwitch™ custom tetramer-neoepitope complex prepared by peptide exchange. The results showed that 0.13% of the CD8-positive T cells in the cultured cells were





Analyzed sample	MFI (X)	% of Exiting peptide (Y)	% Peptide Exchange (100−Y)
Control #2	0.43	0.0	100.0
Control #3	12.60	100.0	0.0
Neoepitope peptide	0.60	1.4	98.6
Wild Type peptide	0.84	3.3	96.7

Figure 4. Preparation of neoantigen-specific tetramers using the QuickSwitch™ custom tetramer kit and confirmation of high-efficiency peptide exchange.

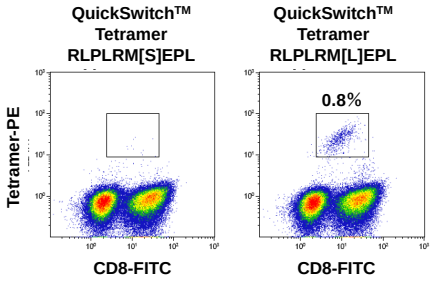


Figure 5. Detection of neoantigen-specific T cells in the peripheral blood of a cancer patient.

tetramer-positive. The tetramer-positive cells were isolated by cell sorting to evaluate the clonality of the neoantigen-reactive T cells. RNA was extracted from the isolated T cells, and cDNA was prepared. The TCR genes were amplified, and the sequence of the CDR3 regions were analyzed using NGS. The sequences of the CDR3 of the TCRβ chain revealed that the neoantigen-reactive T cells obtained from this donor were not monoclonal, but consisted of several clones (oligoclonal) (**Figure 6**).

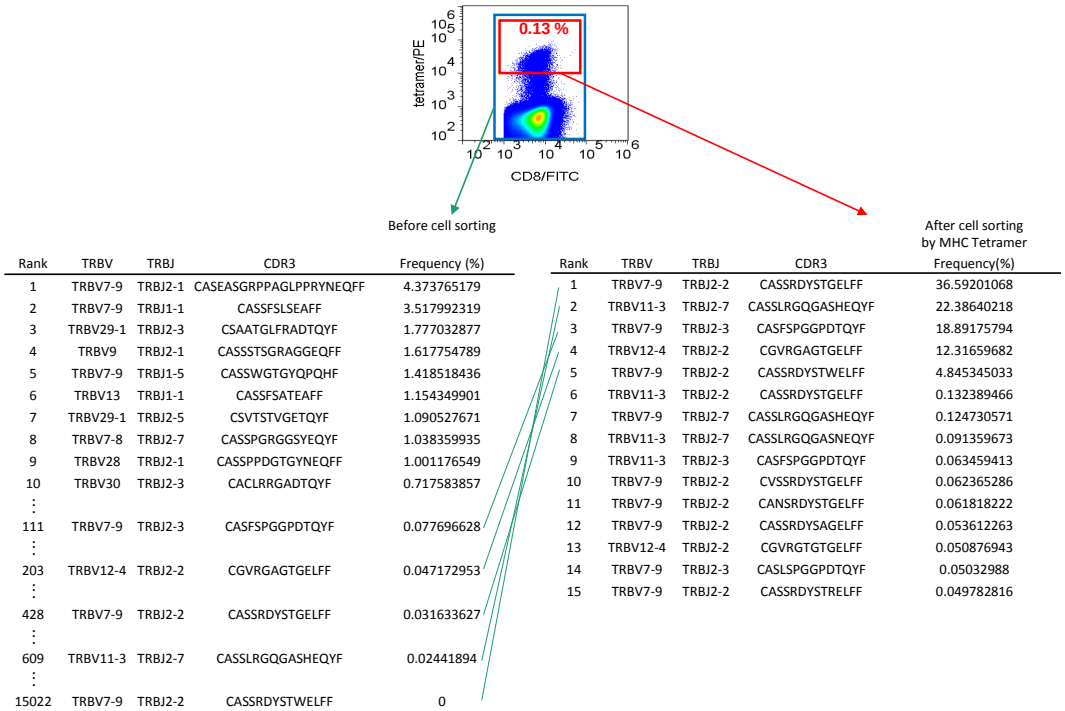
The neoantigen-reactive T cells in the peripheral blood were below the detection limit in the TCR gene analysis using NGS. Even after the in vitro stimulation and expansion with the peptide, no clone reached at frequency of 0.1%. The tetramer staining and sorting makes it possible to easily isolate and analyze such rare neoantigen-reactive T cells (**Figure 7**).

Advantages of QuickSwitch™ custom tetramer

The challenges in the studies of neoantigens include the following: each tumor carries multiple gene mutations; the mutations are different among individual patients; and not all mutated gene products become neoantigens, because different peptides are bound and presented depending on the patient's HLA, which necessitates screening and validation of multiple candidate epitope peptides unique to each patient.

The tetramer technology developed to detect antigen-specific T cells using tetramers of peptide/MHC complex is an innovative advancement that makes direct detection of antigen-specific T cells possible. However, the complex formation was not trivial, involving reconstitution of three properly refolded molecules (peptide, MHC class I molecule, and β2-microglobulin), and only tetramers that were prepared under optimized conditions with known epitope peptides had been made available. While tetramers for epitopes derived from viruses and known cancer antigens could be obtained, preparing tetramers for unknown epitopes, such as neoantigens of individual patients, remains difficult.

QuickSwitch™ custom tetramer is provided with a pre-loaded exiting peptide, with which the tetramer retains proper 3D structure of the MHC-peptide complex.



Rank	TRBV	TRBJ	CDR3	Frequency (%)
1	TRBV7-9	TRBJ2-1	CASEASGRPPAGLPPRYNEQFF	4.373765179
2	TRBV7-9	TRBJ1-1	CASSFSLSEAFF	3.517992319
3	TRBV29-1	TRBJ2-3	CSAATGLFRADTQYF	1.777032877
4	TRBV9	TRBJ2-1	CASSSTSGRAGGEQFF	1.617754789
5	TRBV7-9	TRBJ1-5	CASSWGTGYQPQHF	1.418518436
6	TRBV13	TRBJ1-1	CASSFSATEAFF	1.154349901
7	TRBV29-1	TRBJ2-5	CSVTSTVGETQYF	1.090527671
8	TRBV7-8	TRBJ2-7	CASSPGRGGSYEQYF	1.038359935
9	TRBV28	TRBJ2-1	CASSPPDGTGYNEQFF	1.001176549
10	TRBV30	TRBJ2-3	CACLRRGADTQYF	0.717583857
⋮				
111	TRBV7-9	TRBJ2-3	CASFSPGGPDTQYF	0.077696628
⋮				
203	TRBV12-4	TRBJ2-2	CGVRGAGTGELFF	0.047172953
⋮				
428	TRBV7-9	TRBJ2-2	CASSRDYSTGELFF	0.031633627
⋮				
609	TRBV11-3	TRBJ2-7	CASSLRGQGASHEQYF	0.02441894
⋮				
15022	TRBV7-9	TRBJ2-2	CASSRDYSTWELFF	0

Rank	TRBV	TRBJ	CDR3	Frequency(%)
1	TRBV7-9	TRBJ2-2	CASSRDYSTGELFF	36.59201068
2	TRBV11-3	TRBJ2-7	CASSLRGQGASHEQYF	22.38640218
3	TRBV7-9	TRBJ2-3	CASFSPGGPDTQYF	18.89175794
4	TRBV12-4	TRBJ2-2	CGVRGAGTGELFF	12.31659682
5	TRBV7-9	TRBJ2-2	CASSRDYSTWELFF	4.845345033
6	TRBV11-3	TRBJ2-2	CASSRDYSTGELFF	0.132389466
7	TRBV7-9	TRBJ2-7	CASSLRGQGASHEQYF	0.124730571
8	TRBV11-3	TRBJ2-7	CASSLRGQGASNEQYF	0.091359673
9	TRBV11-3	TRBJ2-3	CASFSPGGPDTQYF	0.063459413
10	TRBV7-9	TRBJ2-2	CVSSRDYSTGELFF	0.062365286
11	TRBV7-9	TRBJ2-2	CANSRDYSTGELFF	0.061818222
12	TRBV7-9	TRBJ2-2	CASSRDYSAGELFF	0.053612263
13	TRBV12-4	TRBJ2-2	CGVRGTGTGELFF	0.050876943
14	TRBV7-9	TRBJ2-3	CASLSPGGPDTQYF	0.05032988
15	TRBV7-9	TRBJ2-2	CASSRDYSTRELFF	0.049782816

Figure 6. Enrichment of neoantigen-specific T cells using tetramer sorting.

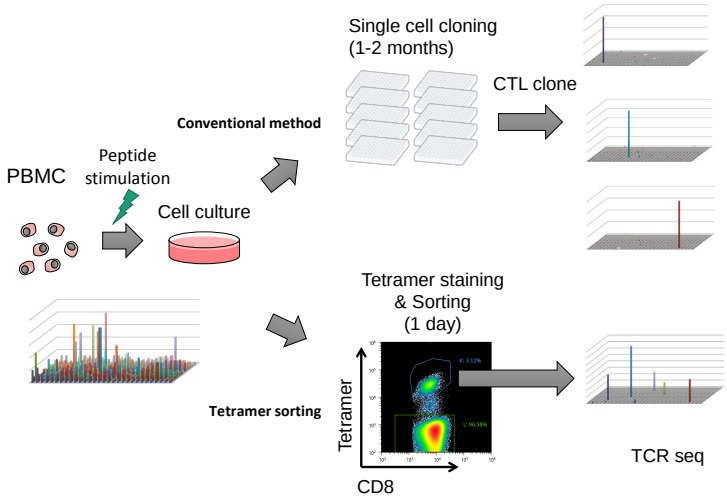


Figure 7. Identification of neoantigen-reactive T cells and TCR analysis using the tetramer.

When a desired neoepitope peptide is added along with the peptide exchange factor, a chemical reaction takes place to exchange the exiting peptide with the desired peptide, yielding a tetramer complexed with the neoepitope peptide. In this procedure, tetramers for multiple neoepitope peptides can be prepared simultaneously, making it possible to build a high-throughput detection system for neoantigen-reactive T cells. QuickSwitch™ custom tetramer will become widely used as an essential tool for studies of neoantigens in the future.

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