PROIMMUNE
www.proimmune.com

ProImmune REVEAL and ProVE®

Rapid Epitope Discovery System

Fast CD4$^+$ and CD8$^+$ T cell epitope discovery, in unique cell-free assays

Simply pass your sequence information to us, select the MHC alleles you are interested in, and within weeks you will receive data on the epitopes that can bind to your chosen MHC alleles. We perform *in vitro* MHC-peptide binding assays, and supply you with the results. For candidate CD8$^+$ T cell epitopes, ProVE® MHC class I Pentamers can be synthesized to allow you to track antigen-specific responses.

Applications

- **T Cell Epitope Discovery**

Map CD4$^+$ and CD8$^+$ T cell epitopes with their MHC restrictions, starting from a complete protein sequence. Alternatively, pre-screen candidate sequences yourself with algorithms or by ELISpot and use ProImmune REVEAL® MHC-peptide binding assays to refine your results.

- **New Information on Known Epitopes**

Extend your knowledge of MHC restriction for a characterized epitope using our large selection of MHC alleles; gain information on binding stability & dissociation kinetics for MHC-peptide complexes.

- **Improve Vaccine Design, Reduce Unwanted Immunogenicity**

Optimize epitopes delivered in novel vaccines to improve efficacy, and eliminate irrelevant or immunogenic sequences to increase vaccine safety.

- **Identify Biomarkers for Clinical Trials**

Identify epitopes associated with a patient response, and use epitope-specific ProVE® MHC Pentamers to monitor antigen-specific T cells in your trial cohort.

- **Find Targets for Immunotherapy**

Delineate T cell epitopes from disease-associated proteins.

- **Investigate Post-Translational Modifications**

ProImmune REVEAL® MHC-peptide binding assays are compatible with modified peptides, such as those containing oxidized or citrullinated amino acid residues.

PROIMMUNE
www.proimmune.com

ProImmune REVEAL and ProVE® Rapid Epitope Discovery System in Detail

The ProImmune REVEAL and ProVE® Rapid Epitope Discovery System follows a very similar outline for identification of MHC class I and MHC Class II epitopes, detailed below:

Peptide Design and Synthesis

A peptide library is designed to span the protein(s) of interest, usually using partially overlapping sequences. Additional controls, suggested by the customer, can also be included for comparison. If a small number of peptides are being screened, then we recommend high-purity custom peptide synthesis, but for larger numbers, it may be more cost-effective to manufacture peptides using our high-throughput PEPScreen™ library system. ProImmune's Immunology Specialists will advise you on the best solution for your assay.

MHC-Peptide Binding Assay

Binding of peptide to MHC is measured, and compared to ProImmune's positive control peptide (an epitope known to bind strongly to a particular MHC). A binding score reported for each peptide-MHC as a percentage of the signal obtained from binding of the control peptide to MHC.
MHC alleles available for testing in ProImmune REVEAL® are shown below

Name	Allele	Name	Allele	Name	Allele
R01	DRA1*01:01 + DRB1*01:01	R32	DRA1*01:01 + DRB1*15:02	P01	DPA1*01:03 + DPB1*01:01
R02	DRA1*01:01 + DRB1*15:01	R33	DRA1*01:01 + DRB1*15:03	P02	DPA1*01:03 + DPB1*02:01
R03	DRA1*01:01 + DRB1*03:01	R34	DRA1*01:01 + DRB1*16:01	P03	DPA1*01:03 + DPB1*03:01
R04	DRA1*01:01 + DRB1*04:01	R35	DRA1*01:01 + DRB1*16:02	P04	DPA1*01:03 + DPB1*04:01
R05	DRA1*01:01 + DRB1*11:01	R36	DRA1*01:01 + DRB3*02:02	P05	DPA1*01:03 + DPB1*04:02
R06	DRA1*01:01 + DRB1*13:01	R37	DRA1*01:01 + DRB3*03:01	P06	DPA1*01:03 + DPB1*05:01
R07	DRA1*01:01 + DRB1*07:01	R38	DRA1*01:01 + DRB5*01:01	P14	DPA1*02:01 + DPB1*01:01
R08	DRA1*01:01 + DRB1*01:02	Q01	DQA1*01:01 + DQB1*05:01	P15	DPA1*02:01 + DPB1*02:01
R11	DRA1*01:01 + DRB1*04:02	Q02	DQA1*05:01 + DQB1*02:01	P16	DPA1*02:01 + DPB1*03:01
R13	DRA1*01:01 + DRB1*04;04	Q03	DQA1*01:02 + DQB1*05:02	P17	DPA1*02:01 + DPB1*04:01
R14	DRA1*01:01 + DRB1*04:05	Q06	DQA1*01:02 + DQB1*06:02	P18	DPA1*02:01 + DPB1*04:02
R15	DRA1*01:01 + DRB1*04:07	Q08	DQA1*03:01 + DQB1*03:02	P19	DPA1*02:01 + DPB1*05:01
R16	DRA1*01:01 + DRB1*04:08	Q09	DQA1*01:02 + DQB1*06:04	P20	DPA1*02:01 + DPB1*06:01
R19	DRA1*01:01 + DRB1*08:04	Q10	DQA1*05:01 + DQB1*03:01	P21	DPA1*02:01 + DPB1*09:01
R20	DRA1*01:01 + DRB1*09:01	Q11	DQA1*02:01 + DQB1*02:02	P22	DPA1*02:01 + DPB1*11:01
R21	DRA1*01:01 + DRB1*10:01	Q12	DQA1*03:01 + DQB1*03:01	P23	DPA1*02:01 + DPB1*13:01
R22	DRA1*01:01 + DRB1*11:02	Q15	DQA1*02:01 + DQB1*03:03	P24	DPA1*02:01 + DPB1*14:01
R23	DRA1*01:01 + DRB1*11:03	Q16	DQA1*03:03 + DQB1*03:03	P25	DPA1*02:01 + DPB1*15:01
R24	DRA1*01:01 + DRB1*11:04			P26	DPA1*02:01 + DPB1*17:01

Table 1: HLA-DR, DQ and DP alleles currently available for the class II HLA-peptide binding assay.
Binding assays are also available for mouse H-2 IAb and H-2 IAd. Blue highlighting shows alleles available for full rate assay.

'Quick-Check' Stability Assay (MHC Class II epitopes only)

For a peptide to perform well as an epitope, it must bind MHC in a stable manner, to facilitate TCR-pMHC interaction. In 'Quick-Check' Stability Assays, binding of peptide to MHC is measured at 0h and at 24h, and the two scores are compared to give a stability index. 'Quick-Check' Stability Assays are available for all HLA Class II alleles.

ProVE® MHC Class I Pentamer Synthesis (MHC Class I Epitopes only)

For each MHC Class I epitope identified in the MHC-Peptide Binding Assay, a MHC multimer (ProVE® MHC Class I Pentamer) can be synthesized by ProImmune, so that T cells specific for the epitope can be identified by flow cytometry.

Pentamers contain 5 MHC-peptide complexes (see figure) and are supplied unlabelled, with a Biotin, APC or R-PE tag for labelling.

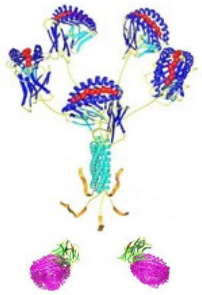


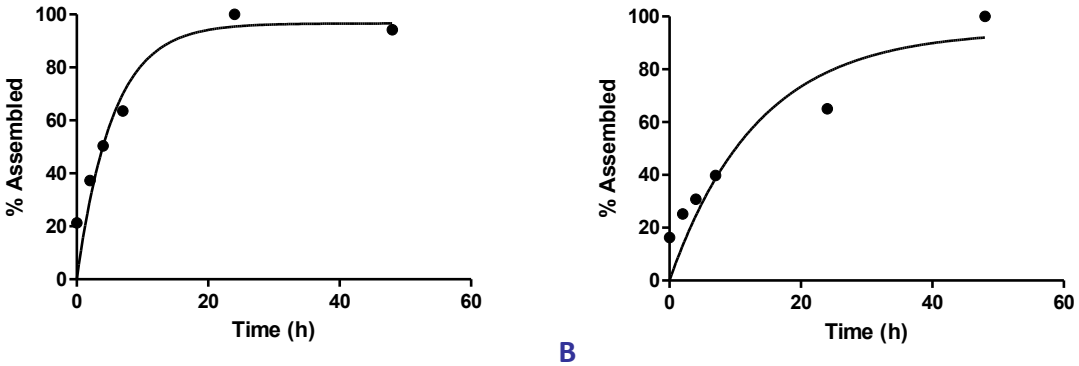
Figure 1: ProVE® MHC Class I Pentamer

Human		Mouse	Rhesus Macaque
A*01:01	B*07:02	H-2Db	Mamu A*01
A*02:01	B*08:01	H-2Dd	Mamu A*02
A*03:01	B*14:02	H-2Kb	
A*11:01	B*15:01	H-2Kd	
A*24:02	B*27:05	H-2Ld	
A*29:02	B*35:01		
	B*40:01		

Table 2: MHC Class I alleles currently available for the HLA-peptide binding assay.

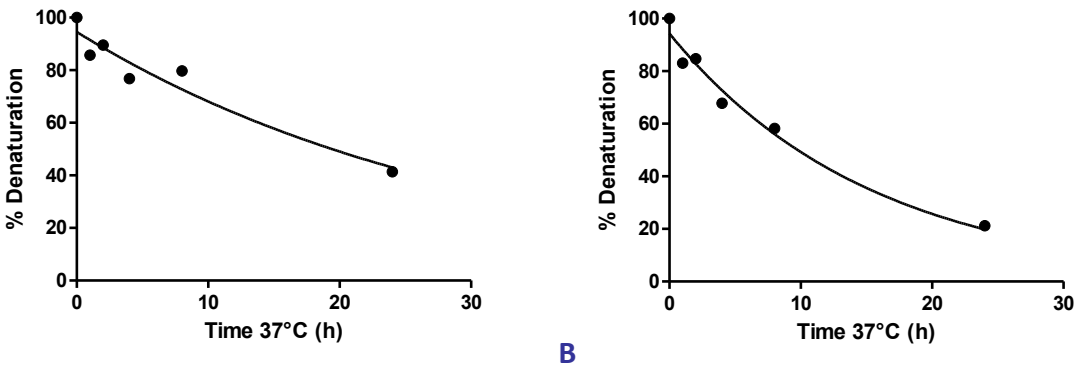
Complete Rate Assay (All MHC Class I alleles, and selected MHC class II alleles)

The on- and off-rates of peptides for binding to MHC are measured at six time points over 48 hours (on-rate) and 24 hours (off-rate), to give detailed kinetics of binding for fully characterizing epitopes. This is available for all MHC class I alleles, and the MHC class II alleles highlighted in blue in table 1.



A B

Figure 2: The on-rate of a test peptide(A) from the human GAD65 protein compared to the on-rate of a control peptide (B).



A B

Figure 3: The off-rate of a test peptide(A) from the human GAD65 protein compared to the off-rate of a control peptide (B).

Reporting

Data are reported in a PDF report and associated spreadsheet files, accessed via our secure server. An Immunology Specialist from ProImmune will be available to review the data with you and answer any questions you may have.

Epitope Validation Studies

Pentamer Staining (for MHC Class I epitopes) or ELISpot (for MHC Class II Epitopes) can be carried out to identify which of the MHC-binding peptides identified using ProImmune REVEAL® perform as epitopes in vivo. These assays can be run by the customer, or alternatively can be run at ProImmune to save time.

Summary

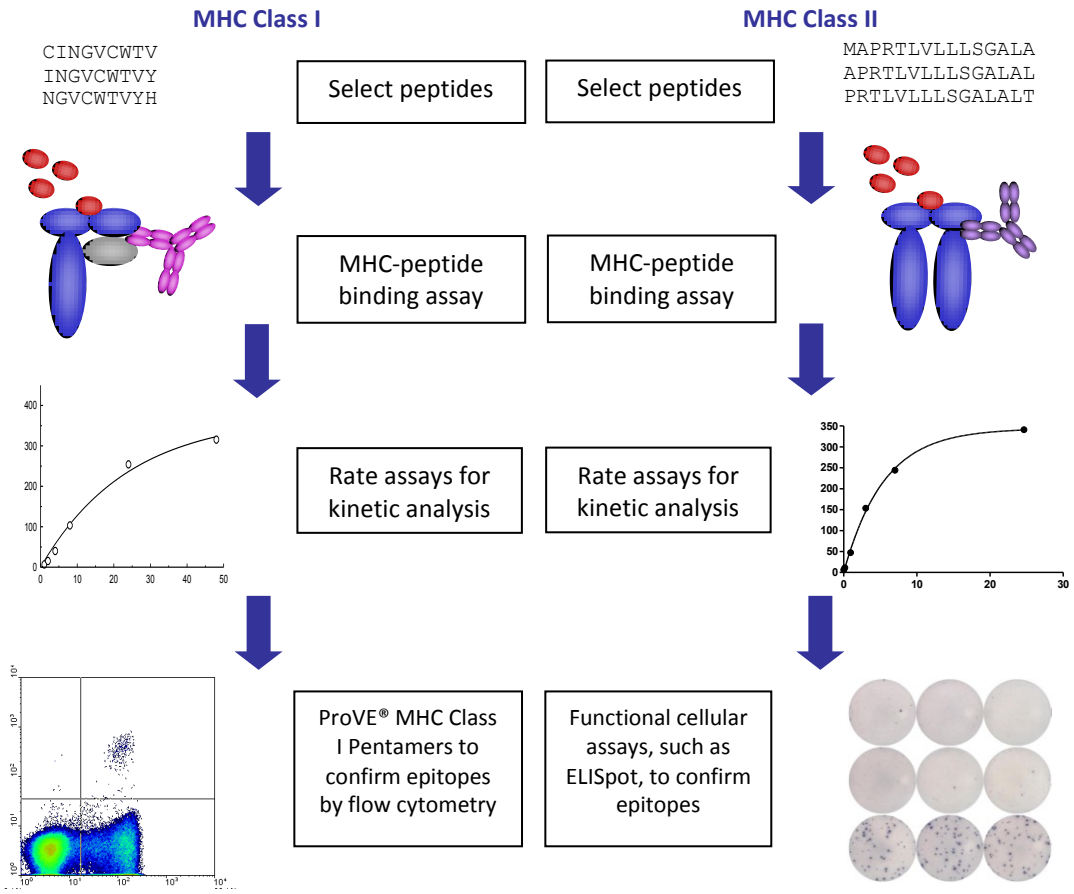


Figure 4: Overview of ProImmune REVEAL and ProVE® Rapid Epitope Discovery System.

Benefits of epitope discovery with ProImmune REVEAL™

- Flexible – order an assay that is as simple or as comprehensive as you need
- Outsourcing saves you time and effort
- Rely on published technology – citations are listed on our website
- MHC restriction of new epitopes is established at the outset
- No need to use valuable cell samples in our cell-free *in vitro* assays
- More reliable and comprehensive data than *in silico* prediction can offer
- ProVE® Pentamers help you to validate results
- Speed – results are with you in just weeks

What our customers say about ProImmune REVEAL™

Prof. Julio Delgado University of Utah, USA
The advantage of ProImmune's *in vitro* system is that we could characterize binding to each HLA molecule individually and not have to guess which of the range of HLA molecules expressed by our patients was responsible for antigen presentation.

Dr. Ruben Varela-Calvino University of Santiago de Compostela (Spain)
I didn't want to ask members of my lab to spend years optimizing an assay which ProImmune runs routinely – time in the lab is precious, particularly for Ph.D students. I've found the service from your team highly professional, the turnaround time for assays very impressive, and I would certainly work with ProImmune again in future.

Dr. Michael Mathis Louisiana State University Health Sciences Center, USA
I've been telling my colleagues that they too should outsource their routine experiments to experts in order to free up their time. It really saves time and money when one take into account the effort needed to set up these assays in house and then wait for results. I will definitely be using the [ProImmune REVEAL™] system again to obtain further data on the hits from the first round of screening.

Dr. Gene Olinger United States Army Medical Research Institute of Infectious Diseases (USAMRIID)
ProImmune accomplished in two months what would have taken three years in our laboratory

Prof. Raymond Dattwyler New York Medical College, New York, USA
I was really happy with the way that these guys took the trouble to adjust my quote so that I had the best assay design for the lowest price. They were really easy to work with, always replied to my questions, and delivered the report on my project on schedule as promised.

PROIMMUNE
www.proimmune.com

Case Study: What causes adverse reactions to Factor VIII treatment?

ProImmune REVEAL™ is used in a major study into the immunogenic epitopes of recombinant Factor VIII

$CD4^+$ T-cell epitopes associated with antibody responses after intravenously and subcutaneously applied human FVIII in humanized hemophilic E17 HLA-DRB1*15:01 mice Steinitz, K. *et al* **Blood** 2012 [PubMedID:22394599]

Patients with severe haemophilia rely on treatment with recombinant Factor VIII (FVIII) to ameliorate their condition and allow them to live normal lives. However, at least a quarter of patients treated with replacement FVIII develop neutralizing antibodies against the 'foreign' protein and this renders the treatment ineffective.

Researchers at Baxter BioScience wanted to understand how these anti-drug antibodies develop, by understanding which portions of the FVIII protein they are targeted against. Antibody-producing B cells require cognate interactions with $CD4^+$ helper T cells and so finding the epitopes presented to $CD4^+$ T cells is essential.

To identify the $CD4^+$ T cell epitopes likely to be important in antibody responses against FVIII, Katharina Steinitz and her colleagues developed a new model mouse. The mouse was missing all of its MHC Class II molecules, and instead expressed a chimeric HLA-DRB1*15:01 molecule, with the human peptide-binding regions and a mouse scaffold.

The team hoped that this mouse would recapitulate human HLA-DRB1*15:01 antigen presentation to $CD4^+$ T cells. When the mice were treated with recombinant human FVIII, some of the cohort did indeed develop anti-FVIII antibodies, and the $CD4^+$ T cells involved in these responses were shown to be polyclonal, just like the $CD4^+$ T cells in humans making an anti-FVIII response.

Having demonstrated that their mouse could indeed mimic a human response to FVIII, the next step was to create hybridoma clones of individual responding T cells, so that the immunogenic T cell epitopes of FVIII could be characterized. Peptide epitopes were identified using a matrix of peptide pools with each responding clone. From over 50 clones isolated, 8 peptides were found to dominate the responses. The mouse results were confirmed using dendritic cells from a HLA-DRB1*15:01-expressing human blood donor. When incubated with FVIII, these dendritic cells could stimulate responses from the mouse T cell hybridoma lines. The same dendritic cells couldn't stimulate a response from T cell hybridoma cell lines from conventional mice, confirming the human DRB1*15:01 specificity of the identified epitopes.

"The Baxter scientists asked ProImmune to test the 6 most common HLA-DRB1 alleles"

The really important question, though, is how far do these results reflect the human population response to FVIII? Only around 8% of the world population expresses DRB1*15:01, and it was important to find out if these 8 new FVIII T cell epitopes could elicit responses in individuals with other HLA types. For this, the team at Baxter used the ProImmune REVEAL™ assay. At ProImmune, we have over 50 different HLA molecules available for testing in our in vitro MHC-peptide binding assays. The Baxter scientists asked ProImmune to test the 6 most common HLA-DRB1 alleles, to investigate how promiscuous binding of their newly-discovered epitopes were. The ProImmune REVEAL™ assay showed that all of the peptides identified could bind to at least 3 of the MHC alleles tested, and detailed measurements of the kinetics of binding showed that many of these interactions were long-lived. Since a stable interaction between peptide and MHC is necessary for a peptide to perform as an epitope, these data suggest that the DRB1*15:01 FVIII epitopes are also likely to be epitopes in patients with a range of other HLA types.

This work marks a great step forward in understanding why haemophilia treatments sometimes fail, and will doubtless inform the research of those at Baxter and in other labs as they seek to reduce the immunogenicity of future treatments.

PROIMMUNE
www.proimmune.com

Case Study: TroVax® Treatment for Colorectal Cancer

ProVE® MHC Pentamers used for validation of novel CD8$^+$ T cell epitopes in colorectal cancer patients vaccinated with TroVax. a novel cancer vaccine

Vaccination of colorectal cancer patients with TroVax® given alongside chemotherapy (5-fluorouracil, leukovorin and irinotecan) is safe and induces potent immune responses. Harrop *et al*. **Cancer Immunology & Immunotherapy** 2008 57: 977-86. [PubMedID: 18060404]

Scientists at Oxford BioMedica have used ProImmune's PEPscreen® peptides and ProVE® MHC Pentamers to detect novel T cell epitopes in patient samples following TroVax® administration. Using ProVE® Pentamers they confirmed the MHC restriction of the T cell epitopes identified and demonstrated that the T cells were truly antigen-specific.

TroVax® consists of a recombinant vaccinia virus (MVA) encoding the tumour-associated antigen 5T4, which is rarely detected on normal tissues but is expressed at high levels on a broad range of solid tumours. The presence of the 5T4 antigen correlates with poor prognosis.

In Oxford BioMedica's Phase I/II trial for colorectal cancer, 94% of patients responded to the TroVax® antigen. A positive correlation was found between the magnitude of the immune response and time to disease progression. Based on this information, it was of great importance for Oxford BioMedica to analyze the 5T4-specific T cell response induced by TroVax® in further detail. In particular, researchers wanted to understand the magnitude, specificity and phenotype of these immune responses.

Two subsequent Phase II studies in metastatic colorectal cancer reiterated the high frequency of responses observed in the earlier Phase I/II. In the immunological analysis of these studies, Oxford BioMedica used a ProImmune PEPscreen® 9mer peptide library in IFN-gamma ELISPOT assays to begin the validation process for 5T4 epitopes implicated in earlier MHC-peptide binding studies. This identified patients who showed no detectable 5T4-specific cellular responses prior to treatment, but who mounted very strong responses following TroVax® vaccination (Figure 5). However, the IFN-gamma ELISPOT assay indicates the frequency of cells that secrete IFN-gamma following peptide stimulation, but does not reveal their phenotype.

Having demonstrated that their mouse could indeed mimic a human response to FVIII, the next step was to create hybridoma clones of individual responding T cells, so that the immunogenic T cell epitopes of FVIII could be characterized. Peptide epitopes were identified using a matrix of peptide pools with each responding clone. From over 50 clones isolated, 8 peptides were found to dominate the responses. The mouse results were confirmed using dendritic cells from a HLA-DRB1*15:01-expressing human blood donor. When incubated with FVIII, these dendritic cells could stimulate responses from the mouse T cell hybridoma lines. The same dendritic cells couldn't stimulate a response from T cell hybridoma cell lines from conventional mice, confirming the human DRB1*15:01 specificity of the identified epitopes.

In order to confirm the nature of the immune responses in patients that had received TroVax®, Oxford BioMedica wished to identify the presence of increased antigen-specific T cell populations in these patients. They turned again to ProImmune for the rapid synthesis of a ProVE® MHC Pentamer Library. ProVE® Pentamers can be generated quickly and affordably for the detection of single antigen-specific T cells in flow cytometry and enable the conclusive validation of new T cell epitopes.

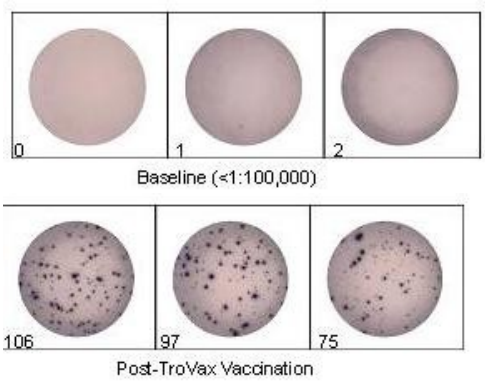


Figure 5: Patients received a total of 6 intramuscular TroVax® vaccinations: 2 before, 2 during and 2 after chemotherapy.

Blood samples were taken prior to the initial vaccination and 2 weeks after completion of chemotherapy. A 96-well culture plate was coated with an anti-IFN-gamma antibody (1-D1K). 200,000 PBMCs were plated per well and incubated overnight at 37°C with 5µg/ml peptide. Cells were removed and the plate washed prior to addition of a biotinylated anti-IFN-gamma detection antibody (7-B6-1). Upon addition of streptavidin-ALP, followed by a precipitating substrate for ALP, spots developed and were counted. The figure shows results for a single patient. Prior to treatment, an average of one spot could be detected per 200,000 cells. However, following a combination of chemotherapy and TroVax® treatment, an average of 93 spots per 200,000 could be detected, indicating that a strong response to 5T4-specific peptide had been mounted.

ProImmune supplied ten 5T4-specific ProVE® MHC Pentamers that were used to validate the responses detected by IFN-gamma ELISPOT and to confirm the MHC restriction of the 5T4 T cell epitopes (Figure 6). The precursor frequencies detected using the ProVE® MHC Pentamers were approximately 2-fold greater than those detected using the IFN-gamma ELISPOT assay against the same peptide antigen, demonstrating a good correlation between these two assays.

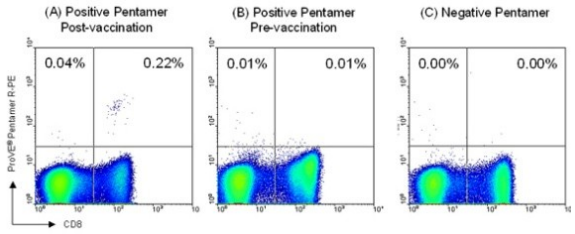


Figure 6: Patient blood samples were taken prior to the initial vaccination and 2 weeks after completion of chemotherapy. For flow cytometry staining, 2 x 10^6 PBMC were incubated with 1 test (0.5µg) ProVE® Pentamer for 10 minutes at room temperature, followed by 1 test R-PE-labeled Pro5® Fluorotag and 1 test FITC-labeled anti-CD8 antibody (clone RPA-T8) for 20 minutes at 4ºC. Samples were analyzed by flow cytometry and 500,000 live events collected. Results are shown for the same patient as in figure 1. A clear population of 5T4-specific CD8+ T cells was detected in the sample taken after completion of chemotherapy using an A*02:01-restricted ProVE® Pentamer (C: 0.22% of live gate). Such antigen-specific cells were not present prior to vaccination with TroVax® (B). No antigen-specific cells were detected at either time-point when an A*01:01-restricted ProVE® Pentamer (negative Pentamer) was used for staining (A).

Administration of TroVax® vaccine clearly elicits potent cellular immune responses in the patient studied, demonstrated by the expansion of antigen-specific T lymphocytes that recognize specific epitopes of 5T4. ProVE® MHC Pentamers provide a powerful means to elucidate a detailed profile of cellular immune responses in patients undergoing immunotherapy. As this study shows, CTL responses to single epitopes can be determined clearly, confirming the applicability of ProVE® MHC Pentamers in the clinical development of new immunotherapies.

Publications Citing ProImmune REVEAL & ProVE®

Bell, MJ. *et al.* (2009) The peptide length specificity of some HLA class I alleles is very broad and includes peptides of up to 25 amino acids in length. **Molecular Immunology**. 46: 1911-1917 [PubMedID: 19157553]

Blancou, P. *et al.* (2007). Immunization of HLA Class I transgenic mice identifies autoantigenic epitopes eliciting dominant responses in type 1 diabetes patients**. Journal of Immunology**. 178: 7458-7466. [PubMedID: 17513797]

Burrows, J.M. *et al.* (2007). The impact of HLA-B micropolymorphism outside primary peptide anchor pockets on the CTL response to CMV. **Eur. J. Immunol.** 37: 946-953. [PubMedID:17357107].

Bui *et al* (2010) Mutation-specific control of BCR-ABL T315I positive leukemia with a recombinant yeast-based therapeutic vaccine in a murine model. **Vaccine** 28:6028 [PubMedID:20619375]

Dang *et al* (2011) Human type 1 diabetes is associated with T cell autoimmunity to zinc transporter 8. **Journal of Immunology** 186:6063 [PubMedID:21471440]

Freed *et al* (2011) Association of the HLA-DRB1 epitope LA(67, 74) with rheumatoid arthritis and citrullinated vimentin binding. **Arthritis Rheumatism** 63:3733 [PubMedID: 22094856]

Harrop, R. *et al.* (2008). Vaccination of colorectal cancer patients with TroVax given alongside chemotherapy (5-fluorouracil, leukovorin and irinotecan) is safe and induces potent immune responses. **Cancer Immunol. Immunother**. 57: 977-86. [PubMedID:18060404]

Lepone *et al* (2010) Monofunctional and polyfunctional CD8+ T cell responses to human herpesvirus 8 lytic and latency proteins. **Clinical and Vaccine Immunology** 17:1507 [PubMedID: 20719985]

MacNamara, A *et al* (2010). HLA Class I Binding of HBZ Determines Outcome in HTLV-1 Infection. **PLoS Pathogens** 6: e1001117 [PubMedID: 20886101]

Muixi, L. *et al* (2008). Thyroglobulin peptides associate in vivo to HLA-DR in autoimmune thyroid glands **Journal of Immunology** 181: 795-807 [PubMedID: 18566446]

Ofran *et al* (2010) Diverse patterns of T-cell response against multiple newly identified human Y chromosome-encoded minor histocompatibility epitopes. **Clinical Cancer Research** 16:1642 [PubMedID:20160060]

Oliveira, ALA. *et al*. (2009). High Frequencies of functionally competent circulating Tax-specific $CD8^+$ T cells in human T lymphotropic virus type 2 (HTLV-2) infection. **Journal of Immunology**. 183: 2597-2965 [PubMedID: 19657093]

Pieper *et al* (2012) α-enolase specific T cells in rheumatoid arthritis – a MHC class II tetramer approach **Annals of the Rheumatic Diseases** 71:A33

Ramaswami, B. *et al.* (2009) HLA-A01, -A03 and -A024 binding nanomeric epitopes in polyomavirus BK large T-antigen. **Human Immunology**. [PubMedID: 19446588]

Robey *et al* (2011) Ex-vivo recognition of late-lytic CD8 epitopes specific for Kaposi's sarcoma-associated herpesvirus (KSHV) by HIV/KSHV-coinfected individuals. **Viral Immunology** 24:211 [PubmedID: 21668362]

Scotto, M *et al* (2012) Zinc transporter (ZnT)8186–194 is an immunodominant CD8+ T cell epitope in HLA-A2+ type 1 diabetic patients. **Diabetologica** [PubMedID:22526607]

Steinitz, K. *et al* (2012) CD4+ T-cell epitopes associated with antibody responses after intravenously and subcutaneously applied human FVIII in humanized hemophilic E17 HLA-DRB1*1501 mice. **Blood** [PubMedID:22394599]

van der Heiden, P. *et al*. (2009). Identification of Varicella Zoster Virus specific CD8 T cells in patients after T cell depleted allogeneic stem cell transplantation. **Journal of Virology** 83: 7361-7364. [PubMedID: 19386715]

Weiskopf, D. *et al.* (2009). Oxidative stress can alter the antigenicity of immunodominant peptides. **J. Leukoc. Biol.** [PubMedID:19801502]

Westrop, S. *et al.* (2009). Novel approach to recognition of predicted HIV-1 Gag B*3501-restricted CD8 T-cell epitopes by $B*3501^+$ patients: Confirmation by quantitative ELISPOT analyses and characterisation using multimers**. J Immunol. Methods**. 341: 76-85 [PubMedID: 19056394]

Email: enquiries@proimmune.com
www.proimmune.com

ST49 v2.0 Issued June 2012