Innovating in Vaccine Discovery

Many diseases with major unmet medical needs today are candidates for vaccine development, including HIV, viral hepatitis, malaria, tuberculosis, haemorrhagic fevers and most forms of cancer. Identification of the antigens that play a role in these conditions and which the immune system can effectively target is critical to design optimized vaccination systems and to monitor the effects of immunization throughout product development.

(Figure 1: false-coloured transmission electron micrograph of Ebola Virus)

Creating a successful vaccine depends on highly detailed understanding of the immune response to a pathogen or to disease-associated proteins. There are many steps in developing a therapy to induce an adaptive immune response against a target that might be either pathogen, such as the agents that cause haemorrhagic fevers, or ‘altered self’ – found in autoimmunity and in cancer.

Generating a ‘good’ antibody response

Often, a starting point for vaccine development is to understand what constitutes a protective antibody response. Which antibodies are protective, and what proteins are they binding to? The magnitude of different patients’ antibody responses (or responses to different formulations, dosing regimens or adjuvants) can be compared using quantitative peptide-protein microarrays. The arrays developed by ProImmune can also be used to identify the epitopes bound by antibodies or by serum samples, and only require a few µl of sample for screening - particularly useful if archive or paediatric samples are being used.

For more information on the memory B cell response elicited by a vaccine, B cell ELISpot is invaluable as a method for determining the number of antigen-specific antibody-secreting cells in a sample. The number of antibody-secreting cells (ASC) present can then be correlated with protection. ProImmune is able to perform B cell ELISpot as a service, to save you the time and cost of optimizing the assay in-house.

T cells as well as B cells

Antibody responses require help from antigen-specific CD4+ T cells, so it’s essential to understand a CD4+ T cell response as part of characterizing a B cell response. Some vaccine strategies are based not on eliciting and antibody response but on boosting the cytotoxic CD8+ T cell response. This strategy holds particular promise in the fight against viral disease, including viral hepatitis. Once the T cell epitopes are
characterized then an optimal vaccine can be designed. T cell epitope discovery can take a great deal of time if functional cellular assays are used to pull out the epitopes from a protein, so this is a strategy best saved for validating candidate epitopes. Ideally, computational biology would be used to predict which peptides from any given protein would bind to MHC molecules and so be presented to T cells, potentially eliciting an immune response. However, algorithms are still in their infancy and may only be informative about the binding and not the kinetics of binding for different peptide epitopes and MHC. It is important to understand binding kinetics, as only peptides which bind stably to MHC are likely to elicit an immune response.

What are the alternatives to functional cellular assays or computational methods?

**T cell epitope discovery**

ProImmune offers various solutions for T cell Epitope discovery. ProImmune REVEAL & ProVE® is a cell-free assay in which candidate peptides are tested for their binding to MHC molecules using our unique technology. In a typical ProImmune REVEAL® study, peptide libraries made from the sequences of whole proteins can be tested against our library of dozens of different MHC molecules. Results are available in just weeks, moving your research forward as fast as possible. We currently have 56 human MHC Class II alleles, 13 human MHC class I alleles, and a range of mouse alleles available for testing.

Alternatively, we have developed ProPresent™ Antigen Presentation Assays. In this assay, immature dendritic cells are loaded with a protein, and the processed and presented peptide epitopes are eluted and identified by Mass Spectrometry (Figure 3). The big advantage of ProPresent™ is that any protein can be loaded into the dendritic cells for analysis, potentially including whole viruses. Again, the process is rapid, and candidate epitope sequences are obtained in a matter of weeks.

A peptide binding to MHC isn’t the end of the story. Not all MHC-presented peptides elicit an immune response, so functional assays are necessary to validate a candidate epitope.

For CD4⁺ T cell epitopes, ProImmune offers fluorescence-based proliferation assays, or T cell ELISpot. CD8⁺ T cell responses can be tested in ELISpot, or using ProImmune MHC Class I
Pentamers to stain patient samples to test for the presence of epitope-specific CD8+ T cells.

**Will it work for everyone?**

Often overlooked at the outset of a study, **HLA** tissue typing can give valuable information about how populations will respond to a vaccine. Population-based data from human samples obtained at the preclinical stage can save valuable development time. ProlImmune’s T cell proliferation assays are designed to provide just this data, as the HLA types of all the samples used are known, and can be selected to cover any test population.

The products and services offered by ProlImmune provide a solution to your epitope discovery needs, whether you need to use us for a particular aspect of programme or choose to outsource a whole project. Our Customer Service Team of Ph.D-trained immunologists can help you design the assays you need. ProlImmune also offers a suite of assays for **Clinical Immune Monitoring**, including ELISpot to GLP/GCP, Intracellular Cytokine Staining, and Flow Cytometry Testing.

[Contact us](mailto:info@proimmune.com) or visit [www.proimmune.com](http://www.proimmune.com) to get started.

---

**Figure 3: ProPresent™ Workflow.** Test proteins are loaded into dendritic cells, and are processed naturally. Presented epitopes are eluted from cell-surface peptide-MHC complexes, and identified by sequencing mass spectrometry.
What do our customers say?

Dr. Michael Mathis
Louisiana State University Health Sciences Center, USA

"My work focuses on the development of adenovirus-based vectors for immunotherapy of breast cancer, using mouse models of the disease. ProImmune's REVEAL™ Rapid Epitope Discovery System has provided a cost-effective method for me to screen my proteins of interest for potentially immunogenic epitopes. I have been particularly impressed with the service that ProImmune offers, so much so that I've been telling my colleagues that they too should outsource more of 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 system again to obtain further data on the hits from the first round of screening."

Dr. Ruben Varela-Calvino
University of Santiago de Compostela (Spain)

Dr. Varela-Calvino has been researching a protein which acts as a target in autoimmune diabetes, and recently benefited from the ProImmune REVEAL™ HLA-peptide binding assays. He and his team wanted to analyze the binding of some candidate peptides from his protein to HLA-DRB1*04:01, one of the major HLA types associated with autoimmune conditions. Dr Varela-Calvino has extensive experience performing HLA-peptide binding assays, and, recalling the length of the process, it wasn’t an experience he was keen to repeat:

"Instead, I performed an internet search, and found ProImmune. 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”.

ProImmune provided HLA-peptide binding assay results in a matter of weeks. His team were quickly able to move on to validating the binding assay data from ProImmune, and were delighted to find that the data they obtained from ELISpot experiments on patient samples correlated almost exactly with their results. As he moves towards collating his data for publication, Dr Varela-Calvino has nothing but praise for ProImmune. He told us "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. 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.”
Case Studies

Case Studies from more of our customers follow, showing just what can be achieved through working with ProImmune. We’ve included studies that illustrate vaccine discovery research programmes, as well as researchers who are using ProImmune’s MHC Pentamers to monitor the responses to vaccines they have developed.

A chickenpox vaccine to target an MHC class I epitope?
Discovery of first VZV HLA-A2 epitope using ProImmune REVEAL & ProVE® technology


Varicella zoster virus (VZV) causes chicken pox in children and shingles in adults, and the virus remains dormant in those it has infected – around 95% of the population. Prior to the work of Pim van der Heiden and colleagues at Leiden University Medical Center a few years ago, there were no characterized MHC class I restricted epitopes identified for VZV. This hampered analysis of cytotoxic T lymphocyte responses in the disease. VZV is a particular problem for patients who experience viral reactivation when they undergo T cell depleting treatments prior to receiving stem cell transplantation, and it was this aspect of the disease which particularly interested van der Heiden et al. They used ProImmune’s HLA-peptide binding assays to determine the binding affinity of peptides from the intermediate-early 62 (IE62) VZV protein.

Selected peptides from the IE62 protein were synthesized by ProImmune as a custom peptide library for analysis in the MHC-peptide binding assays. Peptide binding was compared to that of a known A*02:01 epitope and those that scored higher than this control were identified as putative epitopes and taken forward for further validation using ProVE® Pentamers. ProImmune also included a positive control peptide to confirm assay performance.

One of the peptides identified was validated with Pentamer staining in flow cytometry, and was the first description of an A*02:01 specific response detected for a VZV epitope in patients.

Figure 4: ProImmune REVEAL™ binding data for VZV peptides to A*02:01. Peptide A2-66 (red) is the A*02:01/ALWALPHAA epitope that was further validated using ProVE® Pentamer staining.
The Human Side of Counter-Bioterrorism
T Cell Proliferation Assays Validate Humanization of an anti-VEEV Antibody


Venezuelan Equine encephalitis virus (VEEV) was previously developed as a biological weapon by several nations and it is currently assessed to be an agent with potential for bioterrorist use. In nature, VEEV is a mosquito-borne pathogen that affects equine species but human outbreaks have occurred in Mexico, Colombia, Venezuela and the United States. There is concern that the deliberate release of VEEV would cause human casualties, particularly since no medical countermeasures (vaccines or antivirals) exist.

To address this potential threat, UK Government scientists at Dstl (Defence Science and Technology Laboratory) began work on a virus-neutralising, protective antibody. They used a mouse antibody as the basis for an antibody that could be employed for human treatment.

Introducing a mouse antibody into humans is fraught with difficulty. The human immune system recognizes murine antibodies as foreign and so mounts a response against them. Accordingly, the Dstl Team took one of the most broadly-reactive murine monoclonal antibodies and modified it to remove as much ‘mouse’ sequence as possible. They created two versions, a chimeric mouse-human protein and a fully humanized version created through Complementarity Determining Region (CDR) grafting using human germline IgG framework regions.

Before considering whether to move the treatment into clinical trials, the Dstl team wanted to gather as much information as possible on the potential immunogenicity of their novel antibody construct. For this, they used ProImmune’s Immunogenicity services and requested that ProImmune test both antibodies in CFSE T cell proliferation assays. Briefly, CD4+ T cell proliferation in response to overlapping peptide libraries generated from the sequences of each antibody was assessed in a panel of 40 donors. At the outset, cells are labeled with membrane dye CFSE and this is diluted as the cells divide. The graph shows a summary of responses to each antibody. The results presented show the cumulative antigenicity measured for all study donors. Potential T cell epitopes present in the chimeric antibody have clearly been removed through the humanization process, a demonstrable success.

Figure 5: CD4 T cell proliferative responses to control antigens (PPD, KLH, TT and HA), and peptide libraries derived from the sequences of Dstl’s two antibodies (chimeric, top, and humanized, bottom). The cell division index (CDI) is determined as a ratio of the proportion of CFSE-dim [divided] cells in the stimulated versus unstimulated samples. 40 different donor samples were tested and the cumulative results are shown. Lower scores are indicative of reduced potential antigenicity in humans. Data reproduced by kind permission of Dstl.

In work published in the journal ‘Virology’ the scientists at Dstl showed that the humanized antibody is an effective treatment in VEEV-exposed mice. Even when the antibody was administered 48 hours after viral challenge, antibody treatment was sufficient to allow the test cohort of mice to survive, whilst VEEV-exposed untreated mice succumbed to the virus. If the treatment is to progress, the next step will be to move the humanized antibody to non-human primate models before progressing to human clinical trials. It is possible that this new therapy has the potential to allow a fight back against the consequences of infection with this virus.
Gene Olinger, of the US Army Medical Research Institute of Infectious Diseases (USAMRIID), used the ProImmune Antigen Characterization and Biomarker Discovery Summit to present the very latest thinking on strategy for developing a vaccine for those exposed to filoviruses. There are several challenges to working with Marburg and Ebola viruses: their high infectivity and lethality have obviously impeded basic research, so relatively little is known of their virology, and further, they are highly species-specific. Researchers commonly rely on rodent and macaque models for the diseases, but for filoviruses, bats are strongly implicated as a reservoir species, while higher primates, ungulates and humans will die from the same strain.

There are five strains of Ebola virus, and Gene Olinger is working with the two most lethal, Zaire and Sudan. Ebola belongs to the filovirus family; it is a single-strand RNA virus that produces 7 mRNAs upon infection of a host, including one encoding glycoprotein (GP). GP presents an attractive vaccine target as it is a surface protein, and likely to be involved in cell entry.

In collaboration with Alphavax, scientists in the Olinger lab have been working with a Venezuelan equine encephalitis replicon (VRP) engineered to express filovirus GP. They believe this approach strikes a good balance between vaccine potency and safety. They want to evaluate the antigen components required to generate an immune response in humans. To identify potential T cell epitopes in Ebola Zaire GP, Dr. Olinger chose to use ProImmune’s REVEAL & ProVE® Rapid Epitope Discovery System to analyze the MHC affinity and binding kinetics of a library of Ebola-derived peptides.

![Figure 6: Denaturation curve for a complex of a single Ebola peptide with HLA-A*02:01, measured using the ProImmune REVEAL™ Rate Assay.](image)

As a result of his project using the ProImmune REVEAL® platform, Dr. Olinger has a picture of the regions of Ebola Zaire GP that are likely to cause an immune response in HLA-A*02:01–positive human subjects. Three peptides in particular look to be potentially immunogenic, displaying the strong MHC binding and slow off-rates that, in ProImmune’s experience, are indicative of a good T cell epitope (figure 6).

This information will allow the team to understand the fine detail of an immune response to their vaccine candidate when it comes to be tested. Since ultimately they need to correlate an immune response with protection, this is useful information indeed. The Olinger lab’s next step will be a move into animal models with the newly identified epitopes. Ultimately they are working on developing immune assays to predict protection in vaccinated macaques that can be applied to human vaccine trials.

Discussing the project, Dr Olinger told us that “ProImmune accomplished in two months what would have taken three years in our laboratory.”

*The information contained in this article does not necessarily reflect the position or the policy of the U.S. Government and no official endorsement should be inferred.*
Understanding HIV progression in HLA-B*35:01 patients

ProImmune REVEAL & ProVE® Rapid Epitope Discovery System used to identify novel T cell epitopes in HIV-1


The genetic profile of an individual, such as the patient’s HLA type, is understood to greatly influence the rate of HIV-1 disease progression. For example, HLA-B35 and -B8 have been associated with an increased rate of disease progression in HIV-1+ individuals. ProImmune’s ProImmune REVEAL & ProVE® technology was used to investigate the HIV-1 Gag-specific CD8+ T cell immune response mounted by HLA-B*35:01 individuals to discover the possible reasons for this rapid advancement in the disease.

Westrop et al used the ProImmune REVEAL™ MHC-peptide binding assay to screen 9-mer peptide sequences from the HIV-1 Gag protein against the HLA-B*35:01 allele. A previously unknown epitope was identified, which may be involved in an immune response in B*35:01-positive individuals. ProImmune synthesized a custom peptide library of forty-four 9-mer HIV-1 Gag peptides. These peptides were assessed for binding to B*35:01 using the HLA-peptide binding assay. The binding affinity of the peptides was compared to a control peptide known to have affinity for the B*35:01 MHC complex. Based on this comparison, twelve HIV-1 Gag peptides were classed as ‘hits’, and these peptides were further analyzed using the Quick Check Off Rate Assay. The rates of dissociation of the MHC-peptide complexes were measured at 0, 2 and 24 hours and the half-lives of the complexes were calculated to give an indication of their overall stability. Two peptides, YPLTSLRSL (YL9) and HPVHAGPIA (HA9) were found to form a stable complex with B*35:01, indicating that these may be potential novel epitopes.

Westrop et al then carried out Pro5® MHC Class I Pentamer staining, which showed a well-defined Pentamer+/CD8+ population for the B*35:01/HPVHAGPIA epitope (figure 7). Additionally, no naïve or central memory T cells specific for the HA9 peptide were found, indicating a lack of proliferative potential in infected HIV-1+ individuals. Further validation with ELISpot assays showed that the HA9 HPVHAGPIA peptide elicited the highest ex vivo response of the peptides studied. Further work will show how the presence of this newly-identified epitope modulates disease progression in HIV infection.
Finding epitopes important for T cell control of Human Herpesvirus 8


Human herpesvirus 8 (HHV-8) is the causative agent of Kaposi’s sarcoma, the most common cancer affecting HIV-infected individuals. Preventing or mitigating the effects of HHV-8 infection would therefore be a major health benefit. CD8+ T cell responses are known to be important for the control of this family of viruses, so Lauren Lepone and her colleagues at the University of Pittsburgh investigated these responses to two HHV-8 lytic proteins (gB and K8.1) and two latency proteins (LANA-1 and K12).

Cytotoxic T Lymphocytes (CTL) responding to these proteins are known to be rare, so the team used their own enhanced ELISpot technique to find reactive CTL. They stimulated PBMC with peptide-loaded autologous monocyte derived dendritic cells for a week before ELISpot assay, then used this approach to map ‘hotspots’ of CTL reactivity in the four HHV-8 proteins. This enabled them to narrow down the candidate regions to define 10 potential novel viral epitopes.

Lepone et al turned to ProImmune’s REVEAL® MHC-Peptide Binding Assays to quantitate the binding of their 10 candidate epitopes to HLA-A*02:01, comparing them with five known epitopes and with positive and intermediate binding controls. The data supported their conclusions that these peptides may act as T cell epitopes, showing binding levels comparable to established epitopes (figure 8).

To further characterize the CTL responses to their epitopes, the team commissioned ProVE® MHC Pentamers for the two epitopes with the highest binding scores, and also three characterized HHV-8 epitopes for comparison. Their staining analysis showed the presence of Pentamer-reactive CTL in eight HLA-A*02:01–positive and HHV-8 seropositive donors tested, for all of their epitopes including the two previously unidentified sequences.

In new, as yet unpublished experiments, the team tell us that they have found that the presence of CD4+CD25+ regulatory T cells is detrimental to a CTL response to HHV-8. Viewed together, their work demonstrates that through characterization of HHV-8 epitopes, using technologies such as those provided by ProImmune, we are gathering information that aids our understanding of HHV-8 pathogenesis and will lead us to a strategy for vaccine development.

This work was carried out at the University of Pittsburgh

Figure 8. MHC-peptide binding assay results. The binding score for each peptide is shown relative to a positive control of a known T cell epitope with very strong HLA A*02:01 binding properties. Binding scores are shown for ten novel epitopes (black bars), five known epitopes (grey bars) and positive and additional control peptides (striped bars). Starred epitopes were made as ProVE® Pentamers. Image reproduced with permission from the American Society for Microbiology
Success for Hepatitis C Vaccine Trials at Oxford University
Novel Adenovirus-Based Vaccines Induce Broad and Sustained T Cell Responses to HCV in Man


Infection with Hepatitis C virus (HCV) can cause debilitating liver disease, and up to 170 million people worldwide carry the virus. Infected individuals will often be asymptomatic for years, so rather than waiting for HCV patients to present with liver disease, researchers are trying to develop a preventative vaccine.

In a recent edition of Science Translational Medicine, Oxford University researchers reported the first clinical trial of a preventative vaccine for HCV based on T cell activation.

Ellie Barnes, Paul Klenerman and their colleagues tested a vaccine designed to elicit a long-lived anti-HCV response from CD4+ and CD8+ T cells.

They worked with adenovirus to provide a sturdy vector for expressing portions of viral proteins. However, most people have at one stage or another been exposed to adenovirus, and will clear the virus before it can start to be effective as a vaccine. To overcome this hurdle, the team developed two different adenoviral vectors based on a rare human adenovirus (Ad6) and a chimpanzee adenovirus (ChAd3).

It was important to avoid parts of the virus that mutate quickly, such as the viral coat, so Barnes et al used targets from the viral innards: non-structural proteins 3, 4 and 5. Regions (‘epitopes’) from these proteins had been characterized as important in immune responses from individuals who had managed to spontaneously clear HCV infection.

The responses from vaccinated individuals were very closely monitored. One aspect of this was monitoring the exact viral epitopes being targeted by the two vaccines. Over the course of the study (up to 52 weeks) the percentage of circulating CD8+ T cells staining with Prolimmune’s Pro5® MHC Class I A*02:01/KSALGINAV or A*01:01/ATDALMTGY HCV NS3 Pentamers in each individual were tracked. The data accumulated clearly showed that the CD8+ T cell response went from barely detectable to a sustained 1% of the CD8+ T cell population, an impressive result.

Overall, the anti-HCV responses induced in the trial subjects were prolonged, and broad in terms of the epitopes they targeted. This is important because it should allow the vaccine to work against different HCV strains, and to remain effective even if some of the targets mutate over time.

Speaking to BBC News just after the work was published, Paul Klenerman was optimistic about the promise of his results, stating "The immune responses we've seen are exciting".

Figure 9: Characterization of epitope-specific T cell responses induced after vaccination with either of the two test vaccines. (A) Staining with A*02:01/KSALGINAV Pro5® Pentamer in a representative subject over the study time course. Gating is on live CD3+ T cells. Percentage pentamer+/CD8+ T cells are shown. (B) Ex vivo tetramer+ CD8+ T cell responses over time in six volunteers who responded to the vaccination. Figure reproduced with permission from Science Translational Medicine.
Pro5® Pentamer central to Hepatitis B vaccine research


In hepatitis B infection, the hepatitis B core antigen (HBCAg) is thought to be a major target for specific cytotoxic T cells (CTLs), making it an attractive candidate for therapeutic vaccine development.

Nyström et al. tested the ability of endogenous HBCAg to prime CTLs, and found it to be surprisingly inefficient. They set out to improve CTL priming by HBCAg, manipulating both codon usage and antigen delivery method. The use of a ProImmune custom Pro5® MHC Pentamer was central to the study. The H-2Kb/MGLKFRQL Pentamer, specific for the HBCAg(93-100) epitope, was used to detect and monitor HBCAg-specific CTL in immunized mice by flow cytometry.

The team made use of a transient transgenic mouse model in which HBCAg is expressed by hepatocytes. In this model, clearance of the antigen-expressing cells is dependent on CD8+ CTLs. Using a Chromium release cytotoxicity assay, it was shown that HBCAg DNA delivered via intramuscular injection can prime functional CTLs in vivo, and that this causes elimination of the antigen-expressing hepatocytes. This result led to the conclusion that intramuscular injection of HBCAg DNA represents a viable strategy for therapeutic vaccination.

Further work demonstrated that the CTL response was improved by codon optimization of the injected DNA, and by in vivo electroporation of injected DNA. HBCAg-specific CTLs generated in response to injection of increasing amounts of antigen were quantified using the MGLKFRQL Pro5® Pentamer (figure 10).

![Figure 10: Codon optimization improves the ability of hepatitis B virus core antigen (HBCAg) DNA to prime high frequencies of HBCAg-specific cytotoxic T lymphocytes (CTLs), when delivered at high doses by in vivo electroporation, as determined by Pentamer staining. C57BL/6 mice (n=5 per group) were primed once with 50, 5, or 0.5 ug of codon-optimized HBCAg (coHBCAg) or wild-type HBCAg (wtHBCAg) DNA, and HBCAg-specific CTLs were determined 14 days later using Pentamer staining. Values are given as percentages of HBCAg-specific CD8+ T cells. © 2010 by the Infectious Diseases Society of America.](image)

Using this method of analysis, Nyström et al. showed that high gene doses, of the order of 5ug of injected DNA, are required to effectively prime HBCAg-specific CTL responses. However at low doses (0.5ug), no discernable Pentamer signal was observed, even though similar experiments with hepatitis C virus non-structural 3/4/A DNA show strong responses at this concentration.

These results led to the conclusion that HBCAg is a relatively poor immunogen. The new data presented here on the performance of HBCAg in priming CTLs will be invaluable to the design and development of vaccine strategies for hepatitis B virus.
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]


