

## A Modular Approach for Epitope Discovery and High-Resolution Profiling of Humoral Immune Responses

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An individual's antibody repertoire represents encyclopedic knowledge of previous infections and misguided immunity to auto antigens. Furthermore, the repertoire might presage disease onset and progression. Detailed knowledge of this repertoire may be useful for the diagnosis treatment and prevention of common infections and diseases which are linked to the immune system, such as autoimmune diseases and cancer.

Here we present a comprehensive and modular three-step antibody response and seromarker profiling workflow. Firstly, multiplexed epitope discovery is carried out; using high content peptide microarrays, thousands of peptides are screened. Next, the hits are confirmed by selective antigen profiling. Applying multiwell peptide microarrays, thousands of samples can be tested. Finally, the biomarkers are validated by peptide ELISA.

### Introduction

Humoral immune response can be displayed by circulating antibodies that recognize a vast repertoire of specific antigens. The epitope pattern of relevant antigens recognized by antibodies is patient-specific and may be altered by disease progression, therapeutic intervention and other circumstances. This "epitope spreading" is observed in cancer, infectious and autoimmune diseases and allergies. Epitope discovery, deconvolution of epitope changes and epitope spreading may lead to new biomarkers, individualized therapeutic vaccine strategies, patient stratification and enable the correlation of humoral immune response with clinical outcome.

High density peptide microarrays have been shown to be efficient tools for mapping the immune response at a single epitope resolution. The assay requires only minute amounts of serum or other patient samples (1 µl per assay) to perform a complete analysis on large panels of antigens. JPT produces peptides for microarrays using the high-throughput SPOT™ technology, enabling fast and efficient production of thousands of peptides. The peptides are immobilized in a chemo-selective and directed fashion onto functionalized glass slides. The resulting PepStar™ peptide array contains purified peptides. For the assay, the array is incubated with a biological sample (blood serum/plasma or saliva). Circulating antibodies that bind specifically to the peptides are detected by a fluorescently labelled secondary antibody. The use of PepStar™ peptide microarrays is not limited to the detection of antibody signatures directed against foreign antigens in infectious diseases and allergies. It can also be used to detect autoantibodies such as those directed to tumor associated antigens.

The correlation of humoral immune responses with clinical effect is an important issue since it represents the gateway to novel diagnostics and therapies. However, achieving this goal is still a demanding task. Herein we outline a flexible and modular biomarker discovery workflow (**Figure 1**) based on PepStar™ peptide microarray technology for high resolution analysis of the antibody repertoire. The data generated for this report from deep epitope mapping of humoral immune responses in human samples can be correlated with clinical phenotypes.

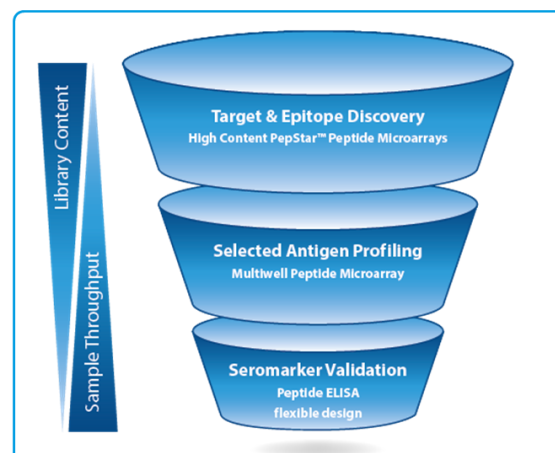
### Material and Methods

Peptide microarray production: peptides derived from EBV-antigens (peptide length 15 amino acids) were synthesized on cellulose membranes using SPOT™ synthesis technology. A reactivity tag was attached to the N-terminus of each peptide and the side chains were deprotected. The peptides were cleaved and eluted from the membrane. QC-measurements using LCMS were performed on random samples of the final library. The peptides were dried and resuspended in printing buffer. Peptide solutions were printed on epoxy-modified slide surfaces using high performance microarray

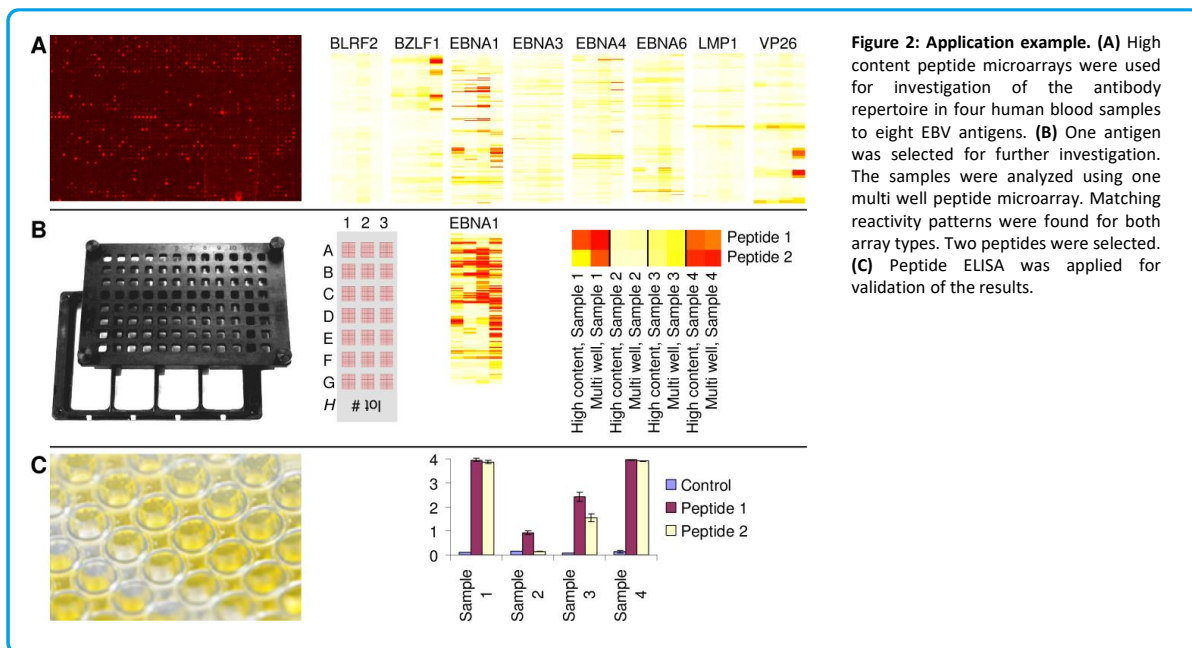
printers. All peptides and controls were deposited in three identical subarrays per block/slide enabling analysis of assay homogeneity and reliability of the results. Peptide microarrays were scanned after the printing process for identification and quality control of each individual spot. Subsequently peptide microarray surfaces were deactivated using quenching solutions, washed and dried. Resulting peptide microarrays were stored at 4 °C until use.

Peptide ELISA Plates: highly purified (>95%) N-terminally biotinylated SPPS peptides were dissolved in peptide coating buffer and incubated in Streptavidin-coated microtiter plates. After washing, the plates were incubated with stabilization buffer. The plates were dried and stored at 4 °C until use.

Immunoassay: human serum samples were diluted in blocking buffer and, after blocking of the surface, incubated on peptide microarrays and peptide ELISA, respectively. After washing, a labelled anti-human IgG secondary antibody was applied. Detection was performed using a microarray scanner or microplate reader, respectively.



**Figure 1: Antibody Response Profiling Workflow.** (1) Multiplexed Epitope Discovery: limited number of samples (~ 20- 100) profiled on high content peptide microarrays with up to 6912 peptides in triplicates. Selection of relevant peptides from thousands of candidate peptides. (2) Selective Antigen Profiling: selected candidate peptides are tested against a large number of samples for verification. On a single multiwell microarray, 21 samples can be tested against up to 192 peptides in triplicates. (3) Marker Validation: Peptide ELISA with highly purified peptides is used as an alternative assay platform for the validation of the results. This platform can be directly transferred into a diagnostic assay.



**Results**

Nearly all adult are infected with EBV. In most cases the infection persists without being recognized. We investigated the B-cell immune response to EBV of four “healthy” adults. We show all of them to be infected with EBV and exhibiting both, common and individual reactivity patterns to EBV antigens.

Four human blood samples were investigated on high content peptide microarrays displaying overlapping peptide scans through major EBV antigens (BLRF2, BZLF1, EBNA1, EBNA3, EBNA4, EBNA6, LMP1, VP26) – in total 1465 peptides. We discovered a remarkable reactivity pattern for the EBNA1 antigen (Figure 2A).

136 peptides derived from EBNA1 were selected for additional experiments. The selected peptides were synthesized and printed on multiwell peptide microarray slides. This format allowed for the testing of all samples on a single slide. The reactivity pattern observed in the first experiment was confirmed (Figure 2B).

Two peptides were selected for further investigation. The samples were tested using peptide ELISA displaying two peptides from EBNA1. Reactivities observed on peptide microarrays were confirmed by peptide ELISA (Figure 2C). Eleven additional human blood samples, of unknown EBV status, were analyzed by peptide ELISA with peptide 1. Consistent with the high prevalence of EBV, ten samples were found to be positive (data not shown).

**Conclusions**

The modular discovery workflow presented here represents an efficient method for detailed mapping of epitopes. Protein based assays measure binding events of circulating antibodies at the protein level. In contrast, peptide microarrays and peptide ELISA provide high resolution information about the antibody repertoire<sup>1,2</sup> down to distinct epitopes. Thus, this approach allows monitoring of individual epitope patterns as well as epitope spreading and addresses natural sequence diversity and post translational modifications. In this communication, we illustrate distinct reactivity patterns detected against ENBA1 in four EBV positive individuals. This valuable information at the sub protein level allows distinction

of, elsewhere homogenous appearing, patients. The presented different assay formats of the workflow can also be performed independently and in any combination for biomarker discovery and validation, patient stratification and companion diagnostic development.

**References**

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2. "Neutralization of porcine endogenous retrovirus by antibodies against the membrane-proximal external region of the transmembrane envelope protein." Waechter et al., J Gen Virol. (2013)

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**The Company**

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